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Two separate functions of class II (Ia) molecules: T-cell stimulation and B-cell excitation

(helper T-cell–B-cell interaction/B-lymphocyte receptors/B-lymphocyte activation/signal transduction)

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ABSTRACT **We have evaluated the role of major histocompatibility complex-encoded class II (Ia) molecules as transmembrane signaling receptors in the T helper cell-dependent activation of B lymphocytes. For these studies, we utilized the murine B-cell lymphoma CH12, which expresses both I-A and I-E class II molecules. In addition, CH12 cells carry IgM of known antigen specificity and require both specific antigen and Ia-restricted T-cell help for the induction of antibody secretion. In this respect, they resemble normal resting B cells. We have studied the ability of antigen-specific or alloreactive T helper cells reactive with either the I-A or the I-E molecules on CH12 to be activated and their ability to stimulate antibody production by CH12. The results show that, although CH12 cells present antigen to T helper cells that interact with either the I-A or the I-E molecules, CH12 cells are stimulated to secrete antibody only by T helper cells reactive with their I-E molecules. Our data demonstrate that class II molecules are transducers of signals for B-cell excitation in addition to serving a restricting function for helper T-cell stimulation. Moreover, the data demonstrate that these two functions, T-cell stimulation and B-cell excitation, are discrete and need not be expressed by the same Ia molecule.**

Helper T (Th) cells are stimulated after recognition of antigen and class II major histocompatibility complex (MHC)-encoded molecules expressed on antigen-presenting cells (APC) (1). Class II (Ia) molecules are, therefore, considered to be "restricting elements," which are required to mediate Th cell activation. In addition to macrophages and other specialized APC, B lymphocytes express Ia molecules and can present antigen to Th cells (2).

Th cell-dependent activation of normal resting (G_0) B lymphocytes also involves the interaction of Th cells with antigen and Ia molecules on the B-cell membrane (3–6). After the binding of antigen by B-cell surface immunoglobulin (sIg), Th cells interact with antigen and Ia molecules and the B lymphocytes are shifted from the resting state. Thereafter, antigen-nonspecific and MHC-unrestricted B-cell stimulating factors are sufficient to promote the growth and differentiation of these "excited" B cells (7, 8). It is not known whether the Ia molecules serve only as restricting elements for Th cell activation during T–B interactions or whether they also serve as transducers of signals for B-lymphocyte excitation.

We have investigated the role played by Ia molecules in B-cell activation, using the CH12 B-cell lymphoma. CH12 cells have sIg specific for a determinant found on sheep erythrocytes (SRBC) and have activation properties similar to resting B lymphocytes (9). These cells have been used previously to demonstrate that the sIg molecule on the B-cell membrane is an activation receptor for B-cell excitation (10). In the current study, we have taken advantage of the fact that CH12 expresses both *I-A*-encoded and *I-E*-encoded class II molecules to determine whether Th cells that interact with these molecules can always stimulate CH12 to antibody secretion. We report here that, although Th cells that interact with either the I-A or the I-E molecules are themselves activated, only the interaction with I-E molecules causes CH12 to produce antibody. These results prove not only that *Ia*-encoded molecules are signaling receptors for B-cell excitation, but also that the two functions of these molecules, Th cell stimulation and B cell excitation, are separate.

MATERIALS AND METHODS

Animals. B10.H-2^aH-4^bp/Wts (2^a4^b) mice were bred at the University of North Carolina. The H-2 haplotype of these mice is *kkkd*, signifying marker alleles in the K, I-A (Aβ, Aα, Eβ). I-E (Eα), and D regions, respectively. All strains will be referred to using this four-letter shorthand nomenclature. Underlining denotes sharing of alleles with those carried by CH12. B10.A (*kkkd*), B10.A(3R) or B10.A(5R) (*bbkd*), B10.MBR (*bkkq*), C3H/HeJ (*kkkk*), and HTI (*bbbd*) mice were purchased from The Jackson Laboratory, or bred in the animal colony at Duke University. B10.A(3R) (*bbkd*), and B10.A(4R) (*kkbb*) mice were kindly provided by Jeffrey Frelinger (University of North Carolina). B10.S(8R) (*kkss*), B10.S(9R) (*sskd*), and B10.TBR2 (*skbb*) mice were provided by Cella David (Mayo Clinic, Rochester, MN).

Th Cells. Alloreactive Th cell lines were enriched in primary mixed lymphocyte cultures as described (11). Keyhole limpet hemocyanin (KLH)-specific Th cell lines were established with T cells purified from draining lymph nodes of immunized mice (12). These T cells were restimulated with irradiated filler cells and antigen once every two weeks. The specificity of each of the lines used in these studies was independently confirmed by restimulation with APC or stimulator cell populations from mice of recombinant haplotypes. KLH-specific Th cells were cloned by using limiting dilution. Spleen cells from mice immunized 4–5 days earlier with SRBC (100 μl of 0.1% suspension) were used as a source of SRBC-primed Th cells (13).

CH12 B Lymphoma Cells. The induction and characterization of the CH12 lymphoma have been described (14). The sIg molecule of CH12 cells is an IgM (μ, κ) with specificity for SRBC. CH12 cells express both the I-A^k and I-E^k class II molecules (9, 14). These cells display activation requirements similar to those of normal resting B cells; specific antigen and H-2-restricted interactions with Th cells are required

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Abbreviations: Th, helper T (lymphocyte); MHC, major histocompatibility complex; APC, antigen-presenting cells; sIg, surface immunoglobulin; SRBC, sheep erythrocytes; KLH, keyhole limpet hemocyanin; pfc, plaque-forming cell(s); IL-2, interleukin 2 (T-cell growth factor).

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to induce their differentiation into antibody-secreting cells (9, 10). CH12 cells can also be stimulated with lipopolysaccharide (LPS) (9). CH12 is maintained as ascites in 2^a4^b or B10.A mice. In some experiments, a clone of CH12 (CH12.6) propagated *in vitro* was used; cloning was achieved by limiting dilution.

Helper Assays and Enumeration of Antibody-Secreting Cells. Cells were cultured exactly as described previously (9, 10). Cultures contained 10^5 CH12 cells per ml and various numbers of alloreactive or antigen- (KLH or SRBC) specific Th cells. In the case of KLH-specific Th cells, KLH was added at various concentrations; SRBC were added at 10^6/ml. Th cells were γ-irradiated [1500 rads (1 rad = 0.01 gray)] before use in all experiments. After 3 or 4 days of culture, CH12 cells were counted and plaqued on SRBC monolayers, using the method of Cunningham and Szenburg (15). Results are expressed as plaque-forming cells (pfc) per ml or as pfc per 10^6 viable recovered CH12 cells on the day of assay; in some experiments the number of pfc are presented as the percent of recovered CH12 cells.

Measurement of T-Cell Activation. T-cell activation was assayed by measuring the release of the T-cell-derived lymphokine, interleukin 2 (IL-2) (12) or T-cell proliferation. T-cell proliferation was measured by [^{3}H]thymidine uptake (11, 12). When normal spleen cells were used as APC, they were irradiated (1500 rads) as described (12). CH12 cells were treated with mitomycin C (50 μg/ml) prior to use as APC (11).

RESULTS

CH12 cells can be stimulated to antibody secretion by using H-2-compatible SRBC-primed splenic T cells and SRBC as specific antigen. Th cells not restricted to MHC-encoded determinants expressed by CH12 did not stimulate the lymphoma, even when APC matching the T-cell haplotype were included in culture, demonstrating that a direct interaction between Th cells and CH12 is required for activation (9). As reported previously (9) and as shown in Table 1, however, not all SRBC-specific Th cells restricted for determinants expressed on CH12 are able to induce differentiation. Th cells from B10.A(4R) mice (*kkbb*) do not stimulate anti-SRBC pfc from CH12. These same B10.A(4R) T cells do stimulate SRBC-specific responses in normal unprimed B cells from H-2^a mice, demonstrating that they include competent Th cells. Previous work has demonstrated that SRBC-primed Th cells from B10.A(3R) mice (*bbkd*) are capable of stimulating an antibody response in CH12 cells (9). Since Th cell interactions occur with *Ia*-encoded molecules on B cells, these data imply that there is a difference in the function of the I-A and I-E molecules on CH12 cells.

To evaluate this difference, alloreactive Th cell lines specific for the I-A, I-E, or both molecules on the CH12 membrane were tested for their ability to induce the differentiation of CH12. As shown in Fig. 1, alloreactive T cells specific for I-A plus I-E or for I-E alone were able to activate CH12. In contrast, I-A-specific Th cells did not induce differentiation at any multiplicity tested. Nevertheless, these I-A-specific Th cells were themselves activated to proliferate to a degree equal to that observed when stimulated with normal allogeneic spleen cells (Fig. 1). This indicates that the failure of these Th cells to stimulate CH12 was not due to their own inactivity.

Table 1. SRBC-primed splenic T cells from B10.A(4R) mice do not stimulate CH12

Source of T cells	H-2 K A E D	CH12 B cells, % pfc*	2^a4^b spleen B cells, pfc/culture
None		0.5 ± 0.2	0
2^a4^b	*k k k d*	2.2 ± 0.3	272
B10.A(4R)	*k k b b*	0.6 ± 0.3	176

SRBC (10^6/ml) were included in all cultures. CH12 were cultured at 10^5/ml; T-cell-depleted 2^a4^b spleen cells were cultured at 10^7/ml. SRBC-primed T cells were irradiated (1500 rads) prior to use. pfc were enumerated on day 3 (CH12) or day 4 (normal spleen cells).
*pfc are expressed as percent of viable recovered CH12 secreting antibody on day 3.

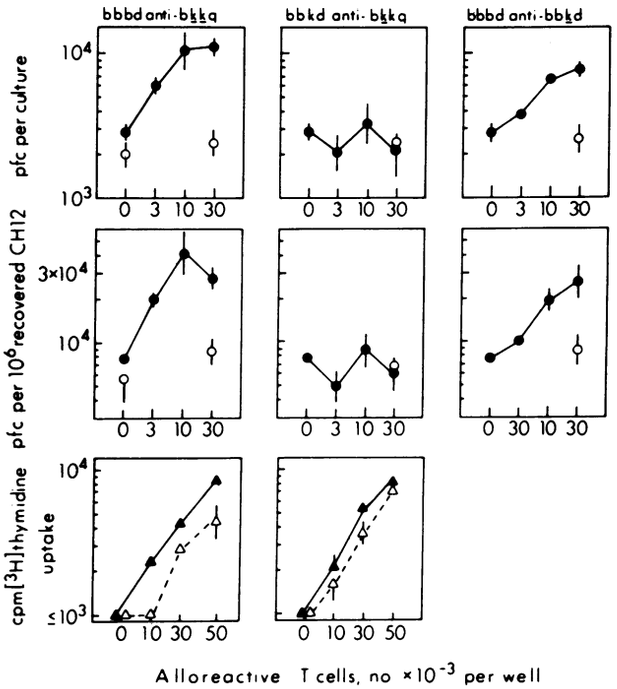

FIG. 1. Alloreactive Th cell-induced differentiation of CH12. CH12 cells were cultured with increasing numbers of alloreactive HTI anti-B10.MBR (*bbbd* anti-*bkkq*), B10.A(5R) anti-B10.MBR (*bbkd* anti-*bkkq*), or HTI anti-B10.A(5R) (*bbbd* anti-*bbkd*) in the presence (●) or absence (○) of SRBC. T cells were irradiated (1500 rads) prior to use. The pfc measured on day 3 of culture are expressed as pfc per culture (*Top*) or pfc per 10^6 recovered CH12 cells (*Middle*). In *Bottom*, the proliferative responses of the Th cells are shown with CH12 cells (▲) or normal B10.A spleen cells (△) as APC. [^{3}H]Thymidine incorporation into proliferating T cells was measured on day 3 of culture. Vertical bars represent standard deviations of replicate cultures.

We next analyzed the MHC-restricted activation of CH12, using KLH-specific Th cell lines derived from B10.A,

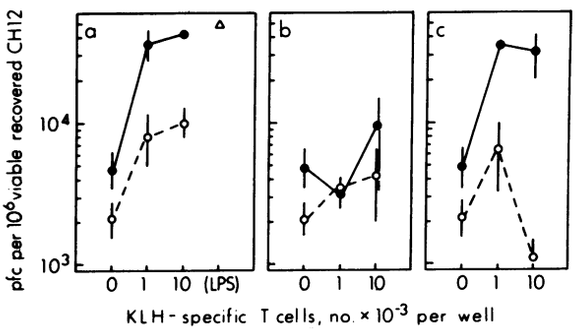

FIG. 2. Activation of CH12 using KLH-specific Th cell lines. KLH-specific Th cells were derived from B10.A (*a*), B10.A(4R) (*b*), or B10.A(3R) (*c*) mice, and increasing numbers were tested for their ability to stimulate CH12 cells in the presence (●) or absence (○) of KLH at 100 μg/ml. All cultures contained 10^6 SRBC. *a* also shows the response of CH12 to *Escherichia coli* lipopolysaccharide (Difco O55:B5) at 100 μg/ml (△). Vertical bars represent SD of quadruplicate cultures.

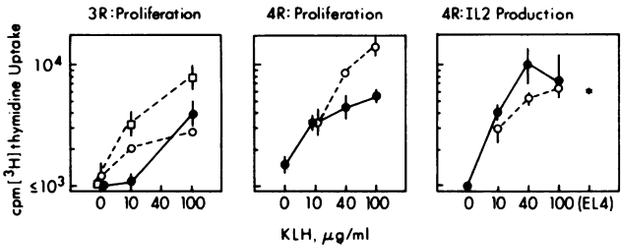

FIG. 3. Activation of KLH-specific B10.A(3R) and B10.A(4R) T cells with CH12 as APC. T cells (3×10^4) were cultured in 200-μl volumes for 3 days with APC and various amounts of KLH. [^{3}H]Thymidine incorporated during the last 6 hr of culture was measured. As APC, 6×10^5 B10.A spleen cells (○), 10^5 CH12.6 cells (●), or 6×10^5 B10.A(3R) spleen cells (□) were used. Prior to labeling with [^{3}H]thymidine, 50 μl of supernatants from cultures containing B10.A(4R) T cells was removed and tested for the ability to support the growth of IL-2-dependent T cells as described (12). A standard source of IL-2 (2% EL4 cell supernatant) is shown as a positive control (*).

Table 2. Antigen presentation by CH12 cells to I-A^k-restricted T-cell clones

Cell line	KLH	[^{3}H]Thymidine uptake, cpm × 10^{-3}		
		B10.A	CH12.6	CH12
Parent line	−	0.6 ± 0.0	0.8 ± 0.2	ND
	+	5.8 ± 0.4	3.3 ± 0.4	ND
Clone E	−	0.1 ± 0.1	1.1 ± 0.3	1.2 ± 0.3
	+	22.0 ± 0.5	11.4 ± 2.0	82.7 ± 8.3
Clone F	−	0.1 ± 0.0	1.2 ± 0.2	2.5 ± 0.4
	+	4.6 ± 0.6	4.0 ± 0.7	21.8 ± 1.7

T cells (10^4 per well) from the parent B10.A KLH-specific T cell line or from each of two I-A^k-restricted T cell clones (E and F) derived from the parent line were cultured for 3 days with 10 μg of KLH per culture and the designated APC in 200-μl volumes. APC were 5×10^5 per culture B10.A spleen cells (irradiated 1500 rads) or 10^4 CH12 ascites tumor cells or the *in vitro* clone CH12.6 (mitomycin C treated). Cultures were pulsed with 0.5 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine during the final 15 hr of culture. Results are mean ± SD; ND, not done.

B10.A(4R), and B10.A(3R) mice, in the presence of KLH and SRBC. As shown in Fig. 2, KLH-specific Th cells of B10.A and B10.A(3R) origins activated the CH12 cells, while those from B10.A(4R) mice did not. Again, the failure to induce CH12 was not due to the lack of activation of the KLH-specific B10.A(4R) T cells (Fig. 3). When CH12 cells were used as APC, the proliferative response of these Th cells and their IL-2 production were similar to the values obtained when conventional sources of APC were substituted (Fig. 3). In this experiment, an *in vitro* propagated cloned line of CH12 cells (CH12.6) was used as a source of APC. Since this clone, unlike the *in vivo* CH12 line, is devoid of contaminating macrophages, these results demonstrate that CH12 cells alone are functional as APC in the activation of B10.A(4R) T cells.

The proliferative response of KLH-specific B10.A(3R) T cells, with various sources of APC, is presented in Fig. 3. These T cells proliferated in response to KLH presented by CH12.6, B10.A APC, and B10.A(3R) APC, although the response to antigen presented by the homologous, B10.A(3R), APC was consistently greater. This was as expected, since only the α chain of the I-E molecule is common to these strains and, therefore, not all of the I-E ($E^b_\beta E^k_\alpha$)-restricted T cells in the B10.A(3R) population recognize the cross-reacting I-E ($E^k_\beta E^k_\alpha$) determinants on CH12.

In the previous experiments (Figs. 2 and 3), KLH-specific T cells from uncloned long-term cell lines were used as a source of T-cell help. Although most of these cells are Lyt-1 positive, and we have never detected Lyt-2-bearing cells (12), we wished to rule out the possible effects of suppressor T cells in the KLH-specific lines as an explanation for the failure of the B10.A(4R) Th cells to stimulate CH12 to produce antibody. We isolated I-A^k-restricted Th cell clones from a KLH-specific B10.A T cell line and tested two of these for their ability to trigger responses in CH12. The specificities of these two clones are presented in Fig. 4. That these two clones were helper cell clones was confirmed by their ability to stimulate anti-SRBC pfc responses in a "bystander" assay (16) with resting B cells from B10.A mice (not shown). Both of these clones proliferated in response to KLH presented by CH12 cells, whether the *in vivo* CH12 line or the CH12.6 subclone was used as APC (Table 2). The higher activity generated with the *in vivo* CH12 might be due to contaminating host macrophages (10), but this has not been evaluated further. In agreement with the results with the long-term B10.A(4R) helper lines, neither clone stimulated CH12 to secrete antibody (Table 3). We conclude that regulatory effects of other cells that may contaminate the Th cell lines do not account for the failure of B10.A(4R) Th cells to stimulate the CH12 B-cell lymphoma. Rather, this is due to the inability of CH12 to be triggered by any Th cell population that does not interact with the I-E molecule.

DISCUSSION

Ia molecules have been well documented as recognition structures for T-cell activation, especially Th cells (1). The data presented in this paper demonstrate a second function, that of transducers of signals for B-cell excitation. This follows from the evidence that, while both the I-A and I-E mol-

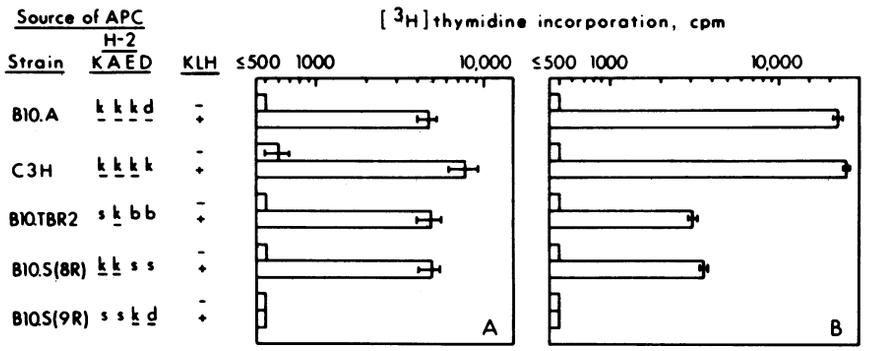

FIG. 4. Proliferative responses of I-A-restricted KLH-specific T-cell clones E (*A*) and F (*B*) to KLH (100 μg/ml) with different sources of APC as shown. Clones were derived from a long-term KLH-specific Th cell line from B10.A mice. Cultures contained 10^4 T cells and 10^6 of the designated APC (irradiated with 1500 rads); proliferation was measured on day 3 of culture. Error bars indicate SD.

Table 3. Failure of I-A-restricted helper T cells to activate CH12

Helper T cells*		Activation of CH12†	
Source	Cells × 10^{-3} per culture	pfc/culture	pfc/10^6 cells
None	—	5,800	3,021
Parent line	3	29,500	32,065
	30	94,200	98,125
Clone E	3	5,800	2,788
	30	1,400	909
Clone F	3	600	242
	30	500	272

*T cells are those described in Table 2; they were irradiated (1500 rads) prior to use. All cultures contained KLH (50 μg/ml) and SRBC (1 × 10^6/ml).

†Activation of CH12 with lipopolysaccharide (100 μg/ml) was 112,800 pfc per culture, 86,769 pfc per 10^6 recovered cells. Anti-SRBC pfc were measured on day 4.

ecules on the CH12 lymphoma are active in presenting antigen to Th cells, only occupation of one, the I-E molecule, led to activation of CH12 and subsequent antibody production. The failure to activate CH12 after recognition of the I-A molecule by Th cells occurred irrespective of Th cell specificity. I-A-restricted Th cells specific for two different exogenous antigens, SRBC and KLH, as well as alloreactive I-A-specific Th cells, were ineffective. Therefore, the failure of these T cells to stimulate CH12 cannot be explained by a conventional *Ir* gene defect. Since both the I-A and I-E molecules are functional for Th cell activation, we conclude that the failure to trigger CH12 cells after the interaction of Th cells with the I-A molecule demonstrates that activation of CH12 via I-E is a direct result of the receptor function of this molecule. The bifunctional nature of class II molecules suggests that, while Ia molecules are ligands for the T-cell receptor in T-lymphocyte activation, T-cell receptors are ligands for the Ia molecule in B-cell excitation.

Various studies on B-cell activation are consistent with the proposed transducer function of Ia molecules. Although occupation of sIg by antigen is normally required for Th cell-dependent activation of resting B cells (10, 17), the requirement for sIg–ligand interactions can be bypassed. Under these conditions, Ia-specific/Ia-restricted Th cells are necessary and sufficient to lead to B-cell stimulation (6, 10, 16, 18, 19), suggesting an excitation function of Ia molecules. Additional evidence for the transducer nature of Ia molecules comes from the work of Palacios *et al.* (20), who demonstrated that monoclonal anti-HLA-DR antibodies can stimulate antibody production from human B cells. We have recently found that monoclonal anti-I-E, but not anti-I-A, antibodies will stimulate CH12 to secrete antibody (ref. 19; unpublished data), indicating that binding of an "inert" ligand (the antibody) to the Ia receptor may be sufficient to activate B cells under certain conditions.

It is possible that the receptor function of Ia molecules is not restricted to B lymphocytes. Since restricted interactions between Th cells and antigen-presenting macrophages result in macrophage activation as well as Th cell activation (21, 22), Ia molecules may have transducer function in these cells as well. Such a role for Ia molecules has previously been suggested by Larsson *et al.* (29), and evidence supporting this hypothesis was recently reported (30).

The inability to stimulate CH12 by using I-A-restricted Th cells raises the intriguing question of whether CH12 is a general model for normal B-cell activation. There is no doubt that some B lymphocytes can be stimulated by I-A-restricted Th cells, but whether these are distinct from those B cells triggered by I-E-restricted T cells is unknown. An alternative view is that the I-A molecule on CH12, while easily recognizable by monoclonal antibodies (9, 14) and capable of serving as a restricting element for Th cell activation, is in some way aberrent. One possibility is that the I-A molecule has a defective cytoplasmic carboxyl terminus that does not allow its normal transducer function to be expressed.

Certainly, occupation of Ia and sIg receptors is not always obligatory for B-lymphocyte stimulation. Various methods have been shown to result in B-lymphocyte proliferation and differentiation *in vitro*, some of which bypass the requirement for sIg receptor–ligand interactions (6, 10, 16, 18, 19), Ia receptor-Th cell interactions (23), or both (5, 7). Indeed, several recent reports (24–26) have demonstrated that MHC-unrestricted Th cell-derived helper factors are apparently able to stimulate resting B cells. We suggest that, rather than being inconsistent with our data and contradicting the proposed transducer function of Ia molecules, these data reflect another of the redundant methods that result in B-cell activation *in vitro*. Whether these various methods also function *in vivo* is not known, but it is possible that the high concentrations of antigen and high multiplicities of Th cells required to effect bystander responses *in vitro* are never realized under *in vivo* conditions (27). We suggest a minimal model for physiologic B-cell activation, in which hapten-carrier conjugates bridge Th cells and B cells (28), making possible the simultaneous occupation of two transducer molecules on B cell membranes, sIg by antigen (10, 17) and Ia by Th cell receptors.

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