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A Primitive T Cell–Independent Mechanism of Intestinal Mucosal IgA Responses to Commensal Bacteria

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The immunoglobulin A (IgA) is produced to defend mucosal surfaces from environmental organisms, but host defenses against the very heavy load of intestinal commensal microorganisms are poorly understood. The IgA against intestinal commensal bacterial antigens was analyzed; it was not simply "natural antibody" but was specifically induced and responded to antigenic changes within an established gut flora. In contrast to IgA responses against exotoxins, a significant proportion of this specific anti-commensal IgA induction was through a pathway that was independent of T cell help and of follicular lymphoid tissue organization, which may reflect an evolutionarily primitive form of specific immune defense.

The most abundantly produced immunoglobulin in mammals is IgA, which is secreted mainly across mucous membranes. Mucosal immunization of the intestine with adjuvants (*1*) is highly dependent on costimulation (*2*) and T lymphocyte help (*3–5*) within the organized germinal centers of mucosal lymphoid tissues, such as Peyer's patches. Yet paradoxically, despite ineffective mucosal immunization in mouse strains that are T cell–deficient and in those that lack costimulation, intestinal IgA is present (*2*, *6*, *7*), and specific anti-rotaviral responses can still be induced (*8*). We show here that, in contrast to conventional views, a large proportion of the intestinal IgA against cell wall antigens and proteins of commensal bacteria is specifically induced in response to their presence within the microflora, but independently of T cells or germinal center formation. This T cell–independent IgA is derived from B (mostly B1) lymphocytes that develop in the peritoneal compartment (*9–11*) and is distributed diffusely in the intestinal lamina propria. Thus, there exists an important pathway of specific intestinal mucosal IgA induction against antigens of the commensal intestinal microflora, distinct from conventional T cell–dependent IgA found in serum and secretions.

When analyzing normal C57BL/6 mice, maintained under specific pathogen-free (SPF) conditions, we found that they exhibited no specific serum IgG or IgA on Western blots against cell wall proteins of *Enterobacter cloacae* (*12*), the predominant aerobe contained in our SPF intestinal bacterial flora [10^8 colony-forming units (CFU)/gram feces; *Escherichia coli* 10^6 to 10^7 CFU/g feces]. However, they did have specific secretory IgA in the intestinal washings [Fig. 1, A and B; similar results were found with the *E. cloacae* (ATCC 29941 reference strain) or wild-type *E. coli*]. Induction of specific anti-commensal IgA is dependent on the presence of the intestinal microflora (*13*), as there was no binding with equivalent concentrations of intestinal IgA from germ-free C57BL/6 animals (Fig. 1A). When *E. cloacae* preparations were digested with proteinase K (5 μg/ml, 37°C, 30 min) before electrophoresis or after transfer to nitrocellulose, no antibody binding was seen, indicating that protein target antigens were being detected.

Although C57BL/6 SPF mice had no IgG or IgA antibodies specific for commensal bacteria in serum, normal mice could induce specific IgG but not IgA 14 days after intravenous injection of 10^6 CFU of *E. cloacae* (Fig. 1B). No specific IgG was induced after intravenously introduced infection in T cell–deficient mice [T cell receptor (TCR) $\beta^{-/-}\delta^{-/-}$; (*6*), Fig. 1B]. In contrast, when adult gnotobiotic C57BL/6 mice were recolonized with an SPF commensal intestinal flora, which is known to result in temporary bacte-

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remia by commensal organisms before adaptive mucosal immune responses develop (*13*), serum IgG was primed to *E. cloacae* proteins (Fig. 1B). The importance of bacteria in avoiding systemic penetration of IgA was demonstrated by the fact that in unmanipulated SPF mice with a targeted deletion of IgA (*14*), or in SPF mice with the alymphoplasia mutation (*aly/aly*), which lack IgA expression (*15*), there is evidence of serum IgG priming against a limited repertoire of *E. cloacae* proteins (Fig. 1B). Antibacterial IgM was present at low levels in the serum of C57BL/6 SPF (Fig. 1B) and germ-free mice, which were unaffected by systemic infection. Thus anti-commensal bacterial secretory IgA is normally induced in the intestine in response to the presence of the bacteria in the intestinal microflora and protects the animal from the penetration of commensal species, but it does not appear in the serum either spontaneously or in response to an infection, whereas serum IgG specific against invading bacteria can readily be induced by T cell–dependent pathways after bacteremia.

To confirm that intestinal IgA is definitely being produced both without cognate T cell help and in normal animals kept in gnotobiotic conditions, 21 different $V_{H\alpha}$ complementary DNA clones from the small intestine of TCR $\beta^{-/-}\delta^{-/-}$ mice and 12 from C57BL/6 germ-free mice were sequenced, and all showed in-frame productive $VDJC_{\alpha}$ rearrangements (*16*). We also verified by fluorescence-activated cell sorting (FACS) that TCR $\beta^{-/-}$ $\delta^{-/-}$ mice consistently had less than 0.05% of live cells from splenocytes or Peyer's patch lymphocytes positive for CD3ε, TCRαβ, or TCRγδ. As expected, neither secretory IgA nor any binding to commensal bacterial proteins was detected in the serum or intestinal secretions of μMT mice (Fig. 1A). That intestinal secretory IgA against commensal bacteria was T cell–independent was shown by virtually identical binding of secretory IgA from normal C57BL/6 mice compared with TCR $\beta^{-/-}\delta^{-/-}$ mice, C57BL/6 nudes (Fig. 1A), or anti-CD4–treated C57BL/6 wild-type mice (*17*, *18*). In these T cell–deficient mice, numbers of IgA secreting cells in the intestinal lamina propria were lower by a factor of 2 to 5 compared with those in wild-type mice (Table 1 and Fig. 2). Also, the intestinal plasma cell number of interleukins IL-$4^{-/-}$, IL-$5^{-/-}$, and IL-$6^{-/-}$ mice was within a factor of 2 of wild-type controls, and the secretory IgA binding was normal. However, the intestinal IgA plasma cell numbers in the unmanipulated T cell–deficient mice do not represent the maximal induction of IgA by the T cell–independent pathway. When TCR $\beta^{-/-}$ $\delta^{-/-}$ mice were gavaged with 10^{10} wild-type *E. coli* on alternate days for 28 days, the plasma cell content rose to 16,200 ± 4800 (n = 4) IgA-secreting cells per 10^5 lamina propria lymphocytes (untreated TCR $\beta^{-/-}\delta^{-/-}$ controls 5300 ± 540/10^5, n = 4); in C57BL/6 wild-type mice, there was also a threefold increase in intestinal IgA plasma cells to 36,000 ± 7200/10^5 (untreated C57BL/6 controls 11,200 ± 2200/10^5, n = 4).

The T cell independence of intestinal secretory IgA responses to commensal bacteria suggested that organized follicular lymphoid tissues, including germinal center formation within Peyer's patches, might not be necessarily involved. Tumor necrosis factor (TNF) receptor–$I^{-/-}$ mice with rudimentary Peyer's patches lacking a follicular dendritic network or B cell follicles (*19*) exhibited nearly normal levels of IgA-producing plasma cells within the lamina propria [(*20*); Table 1 and Fig. 2], and intestinal secretory IgA bound normally to commensal bacterial proteins (Fig. 1A). In contrast, in alymphoblastic [*aly/aly* (*20*)] and lymphotoxin (LT) $\alpha^{-/-}$ mice (*21*), which totally lack intestinal lymphostructures containing B cells [that is, they have no Peyer's patches or draining mesenteric lymph nodes (*22*)], IgA-positive plasmacytes were less than 10 per 10^5 intestinal lamina propria lymphocytes (that is, less than 1/100th of those in controls; Table 1), and intestinal secretions showed no IgA reactivity

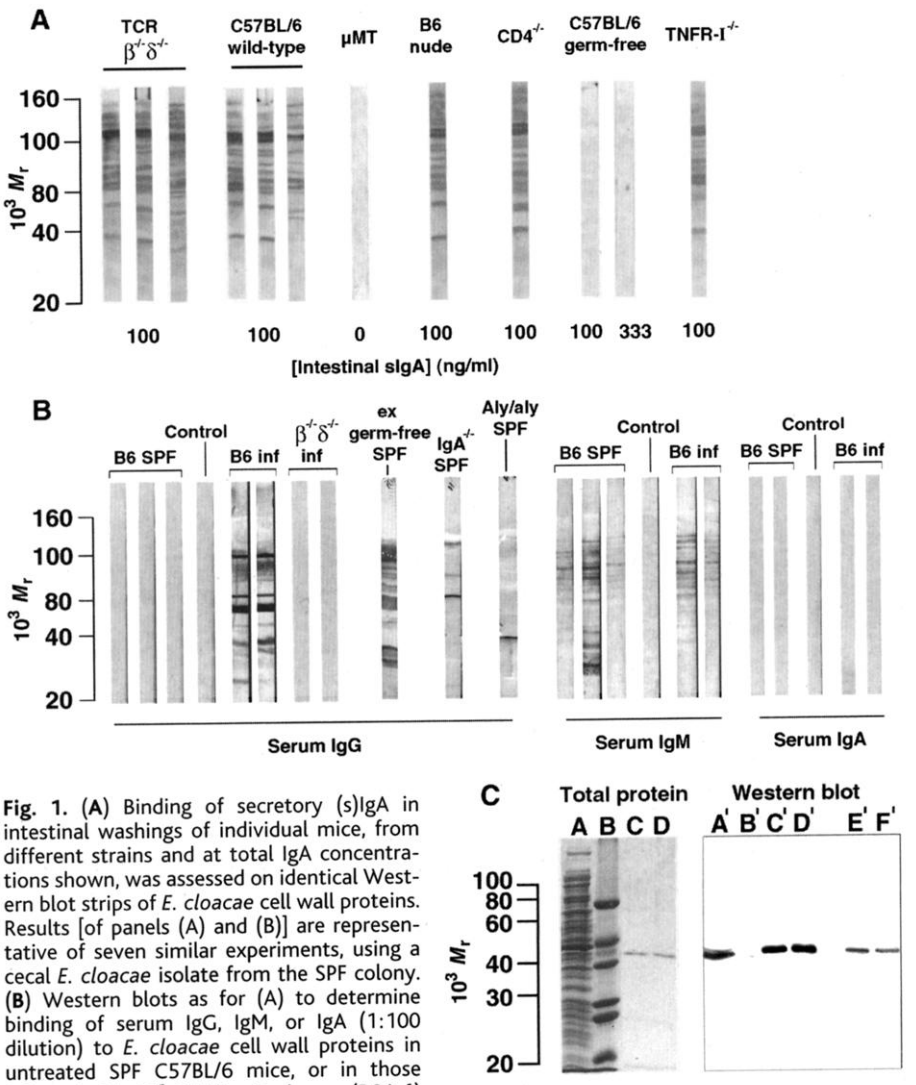

Fig. 1. (**A**) Binding of secretory (s)IgA in intestinal washings of individual mice, from different strains and at total IgA concentrations shown, was assessed on identical Western blot strips of *E. cloacae* cell wall proteins. Results [of panels (A) and (B)] are representative of seven similar experiments, using a cecal *E. cloacae* isolate from the SPF colony. (**B**) Western blots as for (A) to determine binding of serum IgG, IgM, or IgA (1:100 dilution) to *E. cloacae* cell wall proteins in untreated SPF C57BL/6 mice, or in those infected with 10^6 CFU live *E. cloacae* (B6 inf) by intravenous injection 14 days before analysis. Serum IgG binding is also shown for unmanipulated (SPF) IgA$^{-/-}$ and *aly/aly* mice, and after recolonization of adult C57BL/6 germ-free mice with an SPF intestinal flora. (**C**) In vitro purification and in vivo expression of Xa- CAT in intestinal *E. coli*. The chimeric protein Xa-linker peptide-CAT (where the linker peptide was IKAVYNFATCG) was expressed in *E. coli* JM109 on the Pinpoint plasmid after IPTG induction of the *tac* promoter in broth culture, and purified by affinity chromatography. Lanes A to D: SDS-polyacrylamide gel stained for protein with Coomassie blue, lanes A′ to D′: parallel Western blot to detect chimeric protein. Lanes A and A′: total bacterial protein lysate; B and B′: molecular mass markers; C, C′, D, and D′: two fractions of affinity-purified chimeric protein. Lanes E′ and F′: Western blots of cecal sonicates from two mice showing intestinal in vivo expression of the Xa-CAT chimeric protein. Samples were taken 7 days after colonization with wild-type *E. coli* carrying the gene under the control of the *nirB* promoter with induction under the conditions of low oxygen tension in the intestine.

against *E. cloacae*. Thus, although intestinal lymphoid aggregates containing B cells are necessary as an induction site for the distinct intestinal IgA pathway against commensal bacteria, these do not need to be follicularly organized, so germinal center formation, follicular dendritic cells, and antigen-specific B cell–T cell interactions are not essential.

The T cell–independent and organized lymphoid tissue–independent intestinal secretory IgA responses were also seen (*23*) against a highly purified crystallizable preparation of the outer membrane porin protein (OmpF) from *E. coli* (*24*) or against purified lipopolysaccharide (LPS) from *E. cloacae* [(*23*) Fig. 3B] or *E. coli* (O26B6). Intestinal secretory IgA from germ-free C57BL/6 mice bound neither the protein (Fig. 3A) nor LPS (Fig. 3B) components of bacterial cell walls, indicating that colonization of the intestine with the bacterial microflora is prerequisite to induce these specificities of secretory IgA.

We examined whether this pathway of intestinal secretory IgA induction also responds to antigenic changes within an established commensal intestinal flora. Intestinal secretory IgA to a novel antigen could be induced after acute colonization of either C57BL/6 or TCR $\beta^{-/-}\delta^{-/-}$ mice with a wild-type *E. coli* expressing a chimeric protein [(*25*); X_a-chloramphenicol acetyltransferase (Xa-CAT); Figs. 1C and 3C] in vivo as a new component in the flora of otherwise SPF animals. No serum IgA of this specificity was induced after colonization in either wild-type or T cell–deficient animals (Fig. 3C); thus, the specific IgA response is confined to the mucosa. Specific mucosal but not serum IgA responses to engineered mucosal bacteria have also been described after vaginal immunization (*26*). We also found no evidence of systemic T cell priming after intestinal bacterial recolonization of C57BL/6 mice: this was measured when the linker peptide between the Xa-CAT domains was the p57–84 of lymphocytic choriomeningitis virus (LCMV) glycoprotein—this construct normally primes anti-LCMV helper T cell responses after systemic infection (*27*). In contrast, consistent with the well-known T cell–dependent IgA induction found in conventional oral immunization schedules where soluble protein is coadministered with an oral adjuvant (*3*, *4*), when the same (X_a-CAT) purified protein was administered intragastrically in solution with the oral adjuvant cholera toxin, specific intestinal secretory IgA (as well as serum IgA and IgG) was induced only in wild-type mice with functional T cells. [Note that 10 days after the oral immunization schedule (*5*), the anti Xa-CAT midpoint titer corresponded to 1200 ± 800 ng/ml intestinal secretory IgA in C57BL/6 (means ± SD, n = 3); no effective intestinal immunization was detected in TCR $\beta^{-/-}\delta^{-/-}$ mice.] Similarly, we found a specific T cell–independent IgA response to the purified fimbrial protein FimH when the intestines of either C57BL/6 or TCR $\beta^{-/-}\delta^{-/-}$ mice were gavaged with a phase-locked mutant of *E. coli* (AAEC356), which constitutively expresses high levels of type 1 fimbriae. This response is not found with an SPF intestinal flora or after gavage with an afimbriate strain [Web figure 1 (*28*)].

The intestinal IgA-producing plasma cells are known to originate both from the B2 lymphocyte compartment in the bone marrow and from the B1 lymphocytes found in the peritoneum of adult mice (*9*). Peritoneal B1 cells are assumed to be particularly associat-

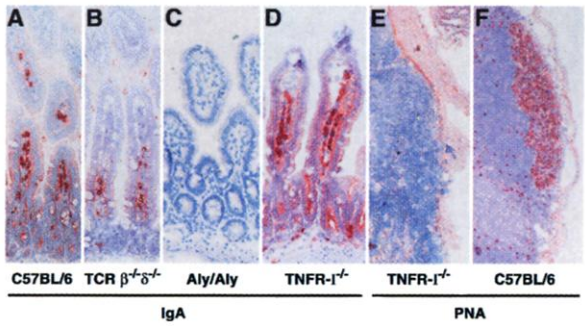

Fig. 2. Immunohistology of small intestinal sections from the indicated strains. (**A** to **D**) Sections stained for IgA. (**E**) is a rare intestinal lymphoid aggregate from a TNFR-$I^{-/-}$ mouse stained for PNA with no germinal center formation. (**F**), Germinal centers are seen in C57BL/6 Peyer's patches.

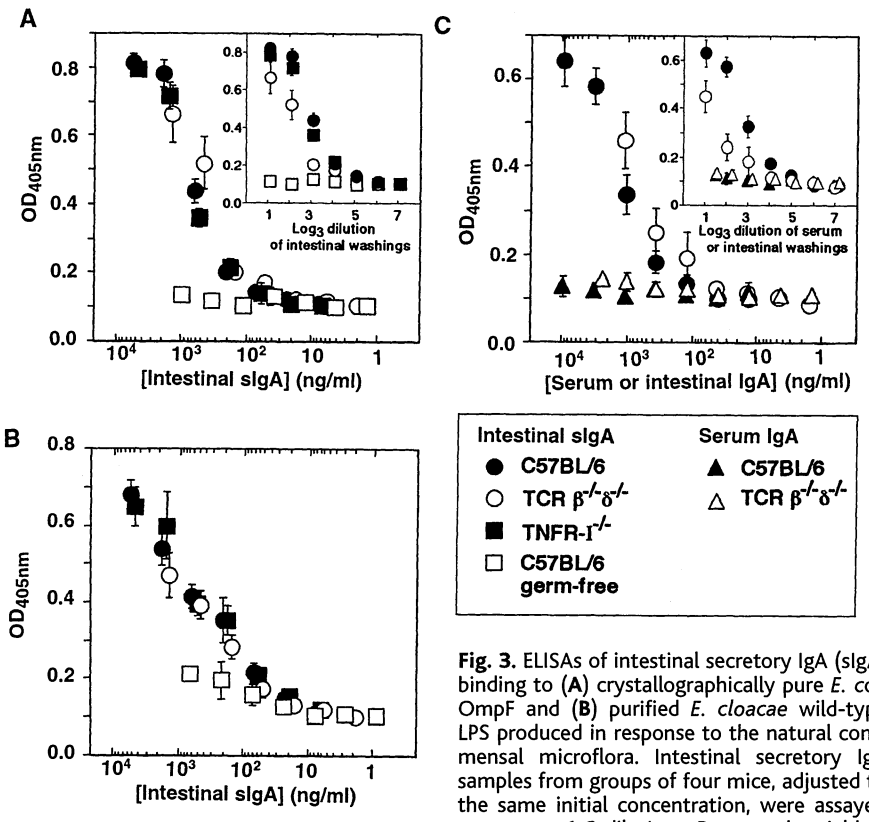

Fig. 3. ELISAs of intestinal secretory IgA (sIgA) binding to (**A**) crystallographically pure *E. coli* OmpF and (**B**) purified *E. cloacae* wild-type LPS produced in response to the natural commensal microflora. Intestinal secretory IgA samples from groups of four mice, adjusted to the same initial concentration, were assayed over seven 1:3 dilutions. Because the yield of intestinal secretory IgA from different strains varies with their different intestinal IgA plasma cell (Table 1), binding data (means ± 1 SD, n = 4) are shown as a function of secretory IgA concentration at each point in the assay. The values expressed as a $\log_3$ dilution of intestinal washings are shown in the inset to (A) as an estimate of overall luminal binding. B cell–dependent (μMT) and *aly/aly* mouse washings contained no secretory IgA and gave no signal in the assays; also, *E. coli* LPS O55:B5 gave results similar to those for the purified *E. cloacae* wild-type preparation. The data illustrated are representative of three experiments. (**C**) ELISA of intestinal secretory IgA or serum IgA binding to purified Xa-CAT chimeric protein 35 days after colonization with wild-type *E. coli* expressing this protein in the intestine in vivo. Binding of intestinal and serum IgA against the chimeric protein in C57BL/6 and TCR $\beta^{-/-}\delta^{-/-}$ mice (four per group) was assessed at 35 days; in addition, no detectable anti-CAT binding was seen in the intestinal IgA of control wild-type mice colonized with bacteria lacking the expression plasmid. An estimate of overall binding is shown in the figure inset with serum dilutions from 1 in 6, although there is inherent dilution of secretory IgA during intestinal washing. Results are representative of three experiments.

ed with the production of natural antibodies of the IgM class against bacterial determinants, and IgA derived from mesenteric lymph node B1 hybridomas has also been found to bind to intact bacteria (*10*). We examined the origin of these T cell–independent IgA-producing B cells using long-term lethally irradiated (9.5 Gy) TCR $\beta^{-/-}\delta^{-/-}$ mice reconstituted with TCR $\beta^{-/-}\delta^{-/-}$ bone marrow together with either TCR $\beta^{-/-}\delta^{-/-}$ or BALB/c B1 peritoneal lymphocytes (*29*). Analysis of the allotypes in these B cell chimeras showed that virtually all the intestinal IgA-producing plasma cells in the TCR $\beta^{-/-}\delta^{-/-}$ mice and the secretory IgA obtained in intestinal washings were of peritoneal B1 origin, whereas serum IgA and bone marrow plasma cells were largely B2 cell–derived (Table 2). The relative contribution of B1 lymphocytes to intestinal IgA is also evident in the *xid* strain, in which the B1 compartment is deficient owing to a mutation in the Bruton kinase. The *xid* mice had reduced intestinal IgA content (2500 ± 1600 secreting cells/10^5 lamina propria lymphocytes, $n = 4$) and evidence of compensatory T cell activation in the Peyer's patches (CD25 44 ± 11%, CD69 62 ± 11%; wild-type controls maintained in identical SPF conditions CD25 14 ± 2%, CD69 10 ± 3% gated on CD4+ lymphocytes), but not in the spleen (CD25 12 ± 2%, CD69 10 ± 3%; wild-type controls CD25 11 ± 2%, CD69 11 ± 1%). Similarly in $CD19^{-/-}$ mice, which also have defective B1 ontogeny, IgA-secreting cells are also reduced (3000 ± 200/10^5), and T cell activation in the Peyer's patches is increased (CD25 28 ± 4%, CD69 55 ± 11%; controls CD25 11 ± 1%, CD69 15 ± 3%).

Taken together, our experiments demonstrate that this special intestinal secretory IgA is produced by a T cell–independent and follicularly organized lymphoid tissue–independent B lymphocyte subpopulation and derived largely from B1 peritoneal cells. Although it has been appreciated that there are low-level IgA responses in the mucosa and serum of T cell–deficient mice after lytic infection with rotavirus when villous architecture is disrupted (*8*), we show here that specific T cell–independent IgA forms part of the normal mucosal response against the luxuriant load of commensal intestinal bacteria. The description of this mucosal T cell–independent IgA induction mechanism against the commensal flora resolves discrepancies between normal intestinal IgA content and ineffective oral immunization with soluble proteins in strains of mice with defective T cell help (*4*). It also explains the enigma of how the lamina propria continues to be resupplied with specific anti-commensal IgA plasma cells in the face of self-limiting germinal center reactions when germ-free mice are colonized with commensal bacteria (*13*). Induction of anti-commensal secretory IgA by this pathway requires the presence of the intestinal microflora and intestinal lymphoid aggregates containing B cells; it is therefore antigen-driven and does not simply reflect the presence of natural antibodies in the sense usually applied to IgM in the serum of naïve or antigen-free animals (*30*). It is well established that different lineages of intestinal T cells have both thymus-dependent and thymus-independent ontogenies (*31*), probably reflecting mucosal T cell pathways that have evolved before and after the evolution of the thymus. Similarly the T cell–independent pathway of mucosal IgA is likely to be evolutionarily ancient compared with T cell–dependent IgA induction. Secretory IgA in the intestine against commensal bacterial determinants induced in a T cell–independent

Table 1. Immunoglobulin A production in wild-type and immunodeficient mice. IgA-secreting cells were measured by ELISPOT assays from intestinal lamina propria lymphocytes and splenocytes ex vivo from the SPF or conventional mouse strains shown. IgA concentrations were measured in parallel on the serum and intestinal washings taken by a standardized technique. All values represent means ± 1 SD ($n \geq 4$; 4 to 17 mice).

Mouse strain	Housing conditions	IgA-secreting cells (no. per 10^5 lymphocytes)		IgA concentration (μg/ml)	
		Intestinal lamina propria	Spleen	Serum	Intestinal wash
C57BL/6	SPF	11,600 ± 1,500	62 ± 16	115 ± 59	18 ± 4.3
TCR $\beta^{-/-}\delta^{-/-}$	SPF	3,900 ± 1,600	11 ± 4	55 ± 33	6.0 ± 3.3
C57BL/6 *nu/nu*	SPF	2,800 ± 1,700	49 ± 13	43 ± 20	5.5 ± 3.8
$CD4^{-/-}$	Conventional	9,100 ± 930	86 ± 27	93 ± 26	15 ± 4.5
TNFR-$I^{-/-}$	SPF	9,500 ± 540	52 ± 26	153 ± 99	14.8 ± 7.1
aly/aly	SPF	<1	<0.1	<0.4	<0.4
$LT\alpha^{-/-}$	Conventional	<10	<0.1	<0.4	<0.4
C57BL/6	Germ-free	1,600 ± 860	14 ± 1	22 ± 4	1.5 ± 0.74

Table 2. IgA origins in long-term T cell–deficient irradiation chimeras. Chimeras were established by irradiation of recipient mice (9.5 Gy) and injection of 1×10^6 bone marrow cells and 1×10^6 purified peritoneal B cells derived from the indicated donors. Peritoneal B1 surface IgM^{hi} $B220^{lo}$ cells were purified by FACS sorting to >99% before injection from pooled donor samples of the indicated strain; equivalent results were obtained by MACS purification of peritoneal cells for secretory IgM^{hi} (not shown). Reanalyzed BALB/c FACS-sorted cells were 82% $CD5^{hi}$, 94% CD11b (Mac-1), and <0.05% CD3ε. BALB/c mice are of the IgA^a allotype, whereas C57BL/6 and TCR $\beta^{-/-}\delta^{-/-}$ mice have the IgA^b allotype. All values represent means ± 1 SD ($n = 3$ to 4 mice) analyzed 6 months after reconstitution. Results are representative of three similar experiments.

Donor		Recipient	IgA-secreting cells (per 10^5cells)						IgA concentration (μg/ml)			
			IgA^a			IgA^{total}			IgA^a		IgA^{total}	
Bone marrow	Peritoneal cavity (B1)		Intestinal lamina propria	Spleen	Bone marrow	Intestinal lamina propria	Spleen	Bone marrow	Serum	Intestinal wash	Serum	Intestinal wash
TCR $\beta^{-/-}\delta^{-/-}$ (IgA^b)	BALB/c (IgA^a)	TCR $\beta^{-/-}\delta^{-/-}$	4,500 ± 750	1.8 ± 0.5	3.5 ± 1.2	3,800 ± 1,200	4.2 ± 0.5	16 ± 7	15 ± 7	5.0 ± 1.9	102 ± 17	5.3 ± 1.5
TCR $\beta^{-/-}\delta^{-/-}$ (IgA^b)	TCR $\beta^{-/-}\delta^{-/-}$ (IgA^b)	TCR $\beta^{-/-}\delta^{-/-}$	<1	<0.1	<0.1	3,000 ± 920	4.4 ± 0.6	23 ± 3	<0.2	<0.2	84 ± 13	4.9 ± 0.9
BALB/c (IgA^a)	BALB/c (IgA^a)	BALB/c	14,860 ± 2,700	22 ± 13	141 ± 46	12,000 ± 4,700	26 ± 4	111 ± 24	171 ± 27	12 ± 5	209 ± 39	14 ± 3.5
B6 (IgA^b)	B6 (IgA^b)	B6	<1	<0.1	ND	9,600 ± 2,300	30 ± 9	ND	<0.2	<0.2	150 ± 17	19 ± 5.6

pathway and independent of follicularly organized lymphoid tissue perhaps offers a glimpse at the primitive, specific antibody–dependent immune system.

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16. Oligo(dT)-primed cDNA was synthesized using polyadenylated messenger RNA from 0.1 to 0.5 g of mid small intestine microdissected free of Peyer's patches. IgA sequences were amplified by polymerase chain reaction (PCR) as described [A. Krebber *et al.*, *J. Immunol. Methods* **201**, 35 (1997)]. Primers (2 μM) were 5′-CGGTGGTTATATCCTTCC-3′ (IgH_α) and a degenerate (HB1–HB19) mix for the V_{H} region. PCR products were purified and subcloned for automated sequencing.
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20. ELISPOT plates were coated with 5 μg/ml of isotype-specific anti-IgA (Zymed) or anti-IgA^a (PharMingen), then blocked and washed [T. Fehr *et al.*, *J. Exp. Med.* **188**, 145 (1998)]. Splenocytes or lamina propria intestinal lymphocytes (5) were diluted 1:5 (six steps starting at 5×10^6 or 2×10^5/ml) in 2% minimal essential medium and incubated for 5 hours (37°C, 5% CO_2). Cells were washed off, and plates were incubated successively with 1:1000 affinity-purified goat anti-mouse IgA (Sigma) and 1:1000 alkaline phosphatase–conjugated donkey anti-goat IgG (Jackson ImmunoResearch) before development of alkaline phosphatase color reactions.
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22. The *aly/aly* mice do contain cryptopatches, which are intestinal lymphopoietic sites for T but not B lymphocytes [H. Saito *et al.*, *Science* **280**, 275 (1998)].
23. Enzyme-linked immunosorbent assay (ELISA) plates were coated with 1 μg/ml of isotype-specific anti-IgA (Southern Biotechnologies) or anti-IgA^a (PharMingen) in 0.1 M $NaHCO_3$ (pH 9.6) overnight at 4°C and blocked with 3% (w/v) BSA at RT. Serum, intestinal washing samples (5), or mouse myeloma IgA^a (PharMingen) were diluted in 100 μl of PBS containing 3% BSA 1:3 over seven to nine steps starting from 1 in 30, 1 in 3, or 3 μg/ml, respectively. Lower dilutions of serum were used in some experiments to compare overall binding between serum and intestinal washings, although the technique of intestinal washing does inherently dilute the secretory IgA sample. After washing, the secondary antibody was goat affinity-purified, peroxidase-conjugated anti-mouse IgA (Sigma; 1:1000 in 100 μl of PBS containing 3% BSA), and the enzyme reaction was developed. Bacterial protein or LPS binding was measured similarly after coating with 1 μg/ml of pure OmpF protein (*24*), 5 μg/ml of pure LPS isolated from cecal wild-type *E. cloacae* [O. Westphal and K. Jann, *Adv. Carbohydr. Chem.* **25**, 83 (1966)] or from *E. coli* O26B6 (Sigma), 1 μg/ml of purified chimeric Xa-CAT or 1 μg/ml of purified FimH (*26*).
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25. Expression of Xa-CAT was from the PinPoint Xa control vector (Promega, Madison, WI) modified by inserting a peptide linker sequence (IKAVYNFATCG) between Xa and CAT between the Hind III and Bam HI sites. For protein purification, the vector was grown in JM109 culture with IPTG induction and subsequent streptavidin affinity chromatography (SoftLink resin, Promega). For in vivo expression the *tac* promoter was replaced between the Cla I and Pst I sites with a *nirB* promoter [L. P. Londono *et al.*, *Vaccine* **14**, 545 (1996)], induced under conditions of reduced oxygen tension. The subcloned construct was transformed without problems of plasmid instability into a wild-type (SPF mouse) isolate of *E. coli* made nalidixic acid–resistant to mark the bacterial chromosome. Mice were gavaged with 4×10^{10} CFU of the transformed bacteria every fourth day for 28 days, and ampicillin was administered with the drinking water.
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29. Recipient mice were irradiated (9.5 Gy) before injection of 1×10^6 bone marrow cells and 1×10^6 peritoneal B cells into the tail vein. Peritoneal B cells (from 10 to 14 mice) were separated by FACS sorting (IgM^{hi}, $B220^{lo}$) and injected in basal salts solution. Chimeras were analyzed after 5 to 6 months.
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32. We are grateful to J. Rosenbusch for supplying purified OmpF protein, to H. Bluethman for TNFR-$I^{-/-}$ mice, to H. Körner for $LT\alpha^{-/-}$ mice, to P. Sebbel and R. Glockshuber for the purified FimH protein, to I. Bloomfield for *E. coli* strain AAEC356, to G. Dougan for advice about in vivo expression, to B. Odermatt for immunohistology and to B. Ludewig, K. McCoy, K. Maloy, and A. Lamarre for helpful discussion.

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Impaired Cued and Contextual Memory in NPAS2-Deficient Mice

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Neuronal PAS domain protein 2 (NPAS2) is a basic helix-loop-helix (bHLH) PAS domain transcription factor expressed in multiple regions of the vertebrate brain. Targeted insertion of a β-galactosidase reporter gene (lacZ) resulted in the production of an NPAS2-lacZ fusion protein and an altered form of NPAS2 lacking the bHLH domain. The neuroanatomical expression pattern of NPAS2-lacZ was temporally and spatially coincident with formation of the mature frontal association/limbic forebrain pathway. NPAS2-deficient mice were subjected to a series of behavioral tests and were found to exhibit deficits in the long-term memory arm of the cued and contextual fear task. Thus, NPAS2 may serve a dedicated regulatory role in the acquisition of specific types of memory.

Because pharmacological inhibitors of gene expression impede learning in a variety of experimental paradigms (*1*), it is anticipated that gene-specific transcription factors may play a regulatory role in learning and memory. For example, mice deficient in cyclic adenosine 3′,5′-monophosphate (cAMP) response element–binding protein exhibit normal learning and short-term memory but are deficient in long-term memory (*2*). The calcium- and cAMP-mediated signal transduction pathways, as well as the transcription factors that alter gene expression as a terminal result of intracellular signaling, are expressed in a wide spectrum of invertebrate and vertebrate cell types (*3*). It is logical to assume that ubiquitous signaling pathways facilitate stimulus-induced changes in neuronal gene expression. Less obvious is how a selective regional pattern of regulatory response may be orchestrated.

The onset of neuronal PAS domain protein 2 (NPAS2) gene expression occurs within the first week of postnatal development, is exclusively restricted to neurons, and is distributed within a stereotypic pattern of fore-

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