Glycolipids as Receptors for Bacillus thuringiensis Crystal Toxin

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Glycolipids as Receptors for *Bacillus thuringiensis* Crystal Toxin

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The development of pest resistance threatens the effectiveness of *Bacillus thuringiensis* (Bt) toxins used in transgenic and organic farming. Here, we demonstrate that (i) the major mechanism for Bt toxin resistance in *Caenorhabditis elegans* entails a loss of glycolipid carbohydrates; (ii) Bt toxin directly and specifically binds glycolipids; and (iii) this binding is carbohydrate-dependent and relevant for toxin action in vivo. These carbohydrates contain the arthroseries core conserved in insects and nematodes but lacking in vertebrates. We present evidence that insect glycolipids are also receptors for Bt toxin.

The crystal (Cry) proteins produced by Bt are pore-forming toxins lethal to insects and nematodes but nontoxic to vertebrates (*1*, *2*). In 2002, more than 14 million hectares of transgenic corn and cotton crops that express Cry proteins were planted worldwide, making these crops safe from specific insect pests and simultaneously resulting in substantial decreases in hazardous chemical pesticide use (*3*, *4*). Cry proteins have now been shown to target nematodes as well, including the intestinal parasite *Nippostrongylus brasiliensis*, suggesting that Cry proteins may be used in the future to control parasitic nematodes of animals and plants (*5*). In the face of the enormous selective pressure generated by widespread use of Cry proteins in crops and organic farming, development of Cry toxin resistance among target populations is considered the major threat to their long-term use (*6*). The ability to detect resistance in the field, which is important for monitoring current resistance-management programs and making corrections before the resistance becomes a widespread problem, relies on molecular and genetic knowledge of the genes and pathways that give rise to resistance. Resistance can be mediated by multiple loci, the identities of which have remained largely elusive. To date, only insect cadherins, which serve as toxin receptors, have been definitively demonstrated to mutate to Cry toxin resistance (*7*, *8*). Other candidates for resistance alleles include a second Bt toxin–binding protein, aminopeptidase N, and a host protease required to process the Bt toxin (*9*, *10*). There are also a number of as yet unidentified loci that can mutate to Cry toxin resistance, including ones important for toxin binding (*11*, *12*).

Using forward genetics, we identified four genes (called *bre* genes for Bt toxin resistant) that mutate to Bt toxin resistance in the nematode *C. elegans* (*13*–*15*). Loss-of-function mutants in this pathway resist at least two Cry proteins, Cry5B, which targets nematodes (Fig. 1A), and Cry14A, which targets nematodes and insects (*13*, *14*). Cry5B and Cry14A are members of the main family of three-domain Bt toxins, which includes the commercially used Cry1, Cry2, and Cry3 toxins (*16*). The *bre* genes encode four glycosyltransferase proteins, act in a single pathway, and are required for the uptake of toxin into intestinal cells, suggesting that they might make a Bt toxin host cell receptor (*13*, *14*). Based on their in vitro activities, the BRE-3 and BRE-5 counterparts in *Drosophila*—EGGHEAD and BRAINIAC, respectively—have been suggested to synthesize the carbohydrate chains present on glycosphingolipids (*14*). We therefore hypothesized that the BRE enzymes might be involved in the biosynthesis of glycosphingolipids and that glycosphingolipids might be heretofore-unrecognized host cell receptors for Bt toxins.

To investigate these possibilities, lipids from wild-type and *bre* mutant animals were extracted, partitioned into two phases, resolved by thin-layer chromatography (TLC), and visualized with the orcinol reagent that stains carbohydrates (Fig. 1B). Wild-type animals contain multiple high-polarity glycolipid species (Fig. 1B, upper phase, components B to F). These glycolipids are ceramide-based (and hence glycosphingolipids) because the carbohydrates can be removed with leech ceramide glycanase (*17*). These upper phase glycolipids are completely absent in *bre-3*, *bre-4*, and *bre-5* mutant animals. In *bre-2* mutant animals, most (B, C, and F) but not all (D and E) upper phase components are missing. In contrast to what was seen in the upper phase, analysis of lower phase (presumably less complex) glycolipids from *bre-4* and *bre-5* mutant animals revealed the appearance of new glycolipid species (Fig. 1B), presumably each representing a different precursor that accumulates as a result of deficiencies in the biosynthetic pathway. Genetic epistasis allows us to infer that the BRE enzymes act in the following order in the synthesis of glycolipids: BRE-3, BRE-5, BRE-4, and lastly BRE-2 [supporting online material (SOM) text], in agreement with the known or proposed activities of these enzymes and the structures of their products. These data demonstrate that BRE enzymes are required to synthesize the carbohydrate chain of glycolipids. The lack of observable defects in protein-linked carbohydrates based on mass spectrometry analysis of N- and O-linked glycans from *bre-3* animals suggests that BRE-3 is not involved in the synthesis of glycoproteins (fig. S5 and table S5). These data and the fact that linkages dependent on *bre-3* and *bre*-5 have been found only in glycolipids indicate that glycolipids and not

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glycoproteins are important for *bre*-mediated Bt toxin susceptibility.

We next tested whether Cry5B can directly bind glycolipids. An overlay technique was used in which crude *C. elegans* glycolipids were fixed in place on TLC plates and then incubated in an aqueous solution of activated, biotinylated Cry5B. After washing away unbound toxin, Cry5B bound to glycolipids was detected by enzyme-linked biotin detection. We found that Cry5B is able to bind to a number of glycosphingolipid species, namely components B, C, E, F, and other minor species (Fig. 2A). Specificity of binding is demonstrated by our observations that neither glycolipid species D (Fig. 2A, lanes 2 and 3) nor the simple glycolipids that accumulate in *bre-4* and *bre-5* mutants (*17*) nor mammalian glycolipid standards (Fig. 2A, lanes 7 and 8) bind Cry5B. As predicted for our resistant mutants, Cry5B-binding glycolipids are missing in *bre-3*, *bre-4*, and *bre-5* mutant animals (Fig. 2A, lanes 4 to 6), and all but one is missing in *bre-2* mutant animals (band E; Fig. 2A, lane 3). Because *bre-2* mutant animals are as resistant as the other mutants (*14*, *15*), expression of band E must not be sufficient for intoxication, perhaps because that glycolipid species is not expressed on the apical surface of intestinal cells.

The binding of Cry5B to purified *C. elegans* glycolipids was confirmed in supported lipid bilayers with microfluidic methods (*18*). Glycolipid component B was purified and incorporated into phosphocholine liposomes at 0.35 mole percent. These liposomes were allowed to form a continuous bilayer in hydrophilic microchannels, and the binding of fluorescently labeled Cry5B was evaluated with total internal reflection fluorescence microscopy. Cry5B binding to component B occurs in a saturable, dose-dependent manner and exhibits an apparent dissociation constant, K_d (±SD), of 0.73 ± 0.06 μM at the particular ligand density tested (Fig. 2B). This K_d falls near the low end of the range observed for many protein lectin–carbohydrate interactions (*19*). No specific binding in the absence of component B was detected. Thus, *C. elegans* glycolipid component B is sufficient to generate specific binding sites for Cry5B toxin in lipid bilayers.

We determined the chemical structures of components B, C, D, and E (Fig. 2C, figs. S1 to S4, and tables S1 to S4). All of these structures contain the core tetrasaccharide *N*-acetylgalactosamine (GalNAc) β1–4 *N*-acetylglucosamine (GlcNAc) β1–3 mannose (Man) β1–4 glucose (Glc), which is an invertebrate-specific glycolipid signature conserved between nematodes and insects but lacking in vertebrates (*20*). Components D and E correspond to the previously described glycosphingolipid structures Nz2 and Nz3, respectively (*21*); the structures of components B and C were previously uncharacterized. The role of BRE-3, BRE-4, and BRE-5 in the synthesis of these structures can be assigned (Fig. 2C) on the basis of epistasis and the predicted or demonstrated biochemical activities of these enzymes (*14*, *22*). We propose that BRE-2 initiates the synthesis of the branched moiety that distinguishes components B and C from D and E (SOM text).

To evaluate the carbohydrate dependence of Cry5B binding to glycolipids, we examined the ability of simple sugars to inhibit the

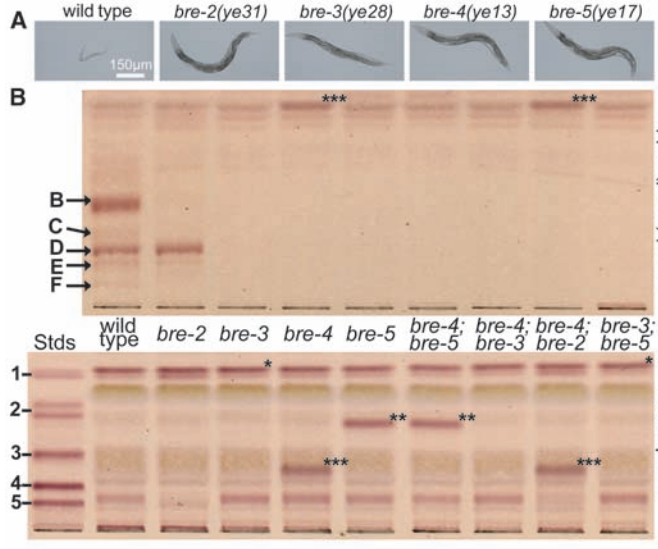

Fig. 1. Bt toxin–resistant animals are defective in glycolipid synthesis. (**A**) Resistance of *bre* mutants to Cry5B is shown after a 3-day exposure of L1 larvae to Cry5B. The alleles shown are used throughout this study. (**B**) Glycolipids were resolved by TLC and stained with orcinol and sulfuric acid. The origin is always at the bottom. Glycolipids stain reddish-brown and contaminating lipids stain yellow or gray. Lipid samples are derived from wild-type animals or from *bre* single- and double-mutant animals, as indicated, and separated as described in (*28*). Glycolipid components are designated as B to F. Numbers to the left denote the saccharide length of glycolipid standards (Stds) at those positions. Asterisks mark intermediates in the pathway where *bre* mutants are presumed to be blocked; the number of asterisks indicates the number of saccharides estimated to exist on the headgroup.

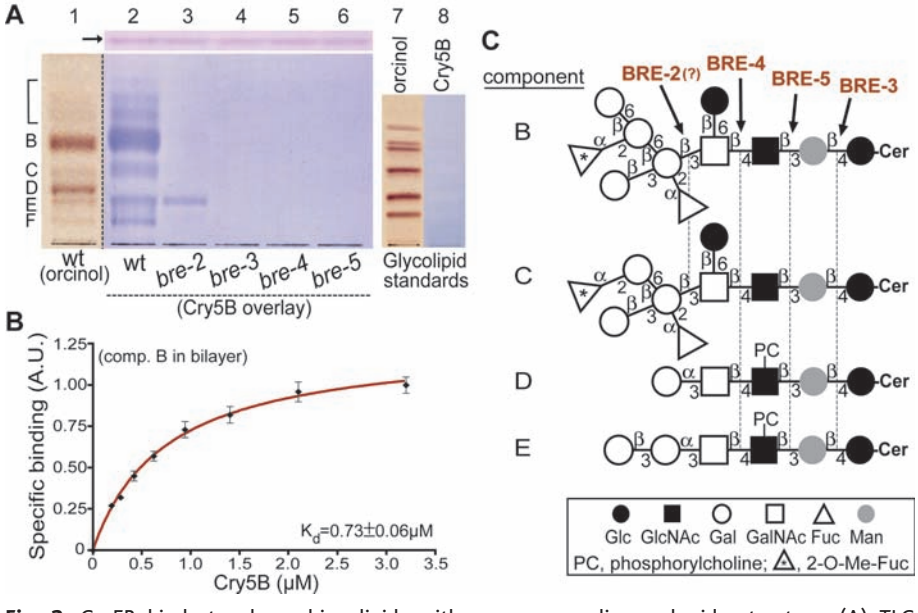

Fig. 2. Cry5B binds to glycosphingolipids with a common oligosaccharide structure. (**A**) TLC overlay reveals the Cry5B-glycolipid interaction. Lane 1, upper phase lipids from wild-type (wt) animals stained with orcinol; lanes 2 to 6, lipids from wild-type and mutant animals overlayed with biotinylated Cry5B; lanes 7 and 8, neutral glycolipid standards stained with orcinol (lane 7) but failed to bind biotinylated Cry5B (lane 8). Bracket indicates additional *bre*-dependent, toxin-binding glycolipids; arrow indicates toxin-independent coloration of a lipid contaminant to verify equal loading. (**B**) Specific binding of Cry5B to glycolipid component B (comp. B) incorporated into a bilayer. Cry5B labeled with Alexa 594 was injected over the membrane at various concentrations and the fluorescence was measured and normalized to data from control bilayers. Data points represent mean specific binding from four experiments; error bars denote standard deviation from the mean. A.U., arbitrary units. (**C**) *bre*-dependent glycolipids are complex ceramide (Cer)–linked oligosaccharides based on a common oligosaccharide core. Structural analysis was performed (*28*), and the glycosidic linkages proposed to be catalyzed by the BRE enzymes are indicated by arrows and dashed lines. Fuc, fucose.

binding of toxin to glycolipids. Glycolipid component B was purified and immobilized in polystyrene wells and then probed with biotinylated Cry5B in the absence and presence of various monosaccharides (Fig. 3A). Galactose is the most potent of the monosaccharide inhibitors, exerting 92 ± 2% binding inhibition at 15 mM. GalNAc also had a significant effect. The galactose analog β-methylgalactoside (conferring 92 ± 4% inhibition at 3 mM) was more inhibitory than the related compound α-methylgalactoside; β-methylglucoside was noninhibitory (Fig. 3B). β-galactose–mediated inhibition also occurred in our microfluidic lipid bilayer system (*17*). Galactose inhibits Cry5B binding to the entire *bre*-dependent glycolipid series in overlay assays (Fig. 3C), suggesting a common galactose-dependent binding mechanism. These data confirm that carbohydrates are key mediators of Cry5B binding to glycolipids and point to the β-galactose–rich terminus of these receptors as an important binding epitope.

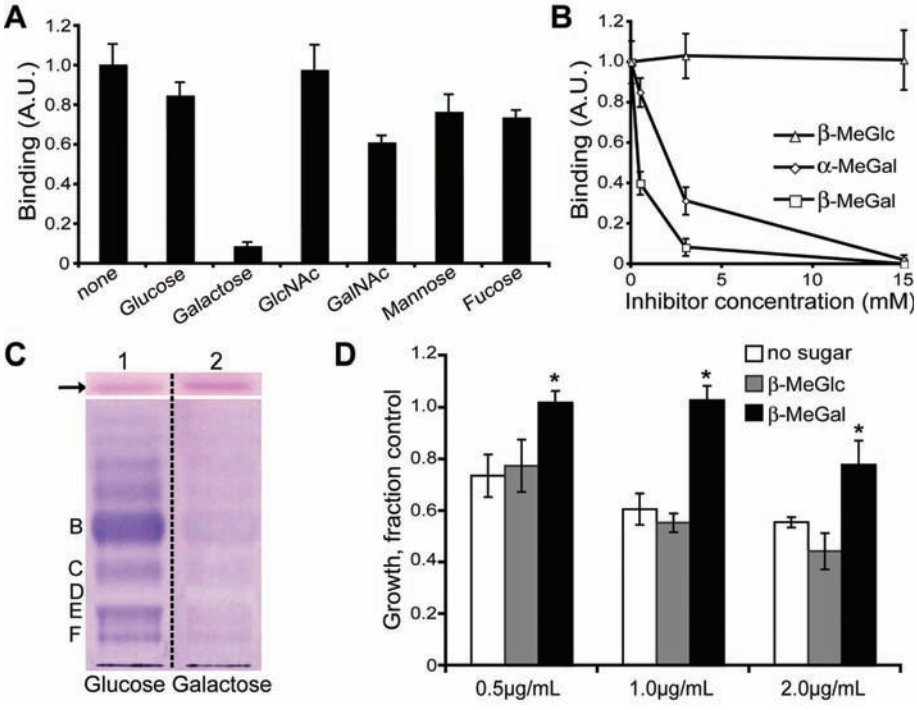

Fig. 3. β-galactose functions in Cry5B-glycolipid binding in vitro and intoxication in vivo. (**A**) Component B was immobilized in polystyrene wells and probed with biotinylated Cry5B in the absence or presence of 15 mM of the sugars indicated. Binding units reflect OD405 units with background subtracted. A.U., arbitrary units. (**B**) Comparison of binding inhibition imposed by anomeric monosaccharide derivatives. Binding was evaluated with the same assay as in (A) but with varying concentrations of inhibitors. β-MeGlc, β-methylglucoside; α-MeGal, α-methylgalactoside; β-MeGal, β-methylgalactoside. (**C**) Galactose inhibits Cry5B binding to all *bre*-dependent glycolipids. Lane 1, binding of biotinylated Cry5B in the presence of 100 mM glucose; lane 2, binding in the presence of 100 mM galactose. Arrow indicates toxin-independent coloration of a lipid contaminant to verify equal loading. (**D**) β-methylgalactoside protects *C. elegans* from Cry5B intoxication. Nematode growth in three doses of Cry5B protoxin is expressed after normalization to no-toxin controls. The test compounds were applied at 15 mM. All values are derived from three independent experiments each testing at least 20 animals. *, $P < 0.05$ versus control β-methylglucoside treatment. Error bars in (A), (B), and (D) denote standard deviation from the mean.

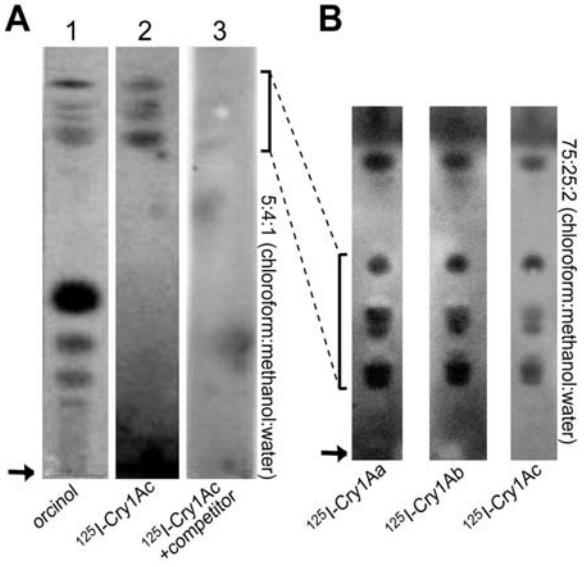

Fig. 4. The insecticidal toxins Cry1Aa, Cry1Ab, and Cry1Ac bind to *M. sexta* glycolipids. (**A**) In each lane, 20 μg of midgut-derived lipids was resolved in 5:4:1 (chloroform:methanol:water). Glycolipids were detected with orcinol (lane 1) or ^{125}I-Cry1Ac overlay (lanes 2 and 3). The experiment in lane 3 included 100 nM of cold Cry1Ac competitor. (**B**) Cry1Aa and Cry1Ab bind to the same *M. sexta* glycolipids as Cry1Ac. Midgut-derived lipids were resolved in 75:25:2 (chloroform:methanol:water), conditions that better separate the cluster of Cry1A-binding glycolipids, and probed with ^{125}I-Cry1Aa, ^{125}I-Cry1Ab, and ^{125}I-Cry1Ac. Brackets indicate Cry1A-binding glycolipids. Arrows indicate origins.

Considering the substantial conservation of *bre*-dependent glycolipids in nematodes and insects and the conservation of Cry toxin structures (including lectin-like domains), it seems likely that insecticidal Cry toxin activity is also modulated by glycolipid host cell receptors. Consistent with this, we found that Cry1Ac toxin binds to glycolipids extracted from the midguts of the tobacco hornworm, *Manduca sexta* (Fig. 4A) (*23*). Competition of binding with unlabeled Cry1Ac indicates that binding is specific (Fig. 4A). Furthermore, Cry1Aa and Cry1Ab toxins bind the same *M. sexta* glycolipids as do Cry1Ac, consistent with glycolipids serving as general host cell receptors for these toxins (Fig. 4B).

Previously, glycolipid levels have been found to be substantially reduced in a Cry1Ac-resistant *Plutella xylostella* strain (*24*). It was proposed in the same report that alterations in glycolipids are involved in the evolution of *P. xylostella* resistance to Cry1Ac, although possible glycolipid receptor functions were not discussed. In a second study, a capacity for nonpurified *Bt* kurstaki toxins to bind to insect glycolipids was shown, but it was postulated that the in vivo toxin receptors were other glycoconjugates, such as glycoproteins (*25*). In addition, we have shown that Cry14A, a toxin

active against both nematodes and insects, requires the *bre* pathway for full activity against *C. elegans* (*13*, *14*). Taken together, our data and these studies suggest that both nematicidal and insecticidal three-domain Bt toxins use invertebrate glycolipids as host cell receptors and that the loss of glycolipid receptors represents an important mechanism for Bt toxin resistance. The ease with which glycolipids can be isolated and analyzed suggests that it will be feasible to monitor glycolipid-mediated resistance in field and laboratory populations of insects and nematodes.

Glycolipids, however, are not the only class of Bt toxin host cell receptors. For insects, high-affinity protein receptors, such as insect cadherins and aminopeptidases, have been shown to play functional roles as Cry1 receptors, although correlating protein receptor defects with binding defects has not always been simple. For example, in at least two cases, Cry1Ac has been found to bind specifically to membranes of cadherin receptor mutants that are Cry1Ac resistant (*26*, *27*), leading to the hypothesis that a multistep binding process involving multiple receptors is required for proper pore formation. For nematodes, our data suggest that although *bre*-dependent glycolipids are important for Cry14A function, there is likely another binding factor involved in Cry14A toxicity (*14*). We hypothesize that glycolipid and protein receptors may both play a role, sequentially or simultaneously, in positioning Bt toxins appropriately at the bilayer or in inserting toxins into the bilayer.

Mammalian cells do not bind three-domain Bt Cry toxins (*2*), and the results presented here provide a plausible molecular basis for the lack of toxicity of Cry toxins toward vertebrates. Vertebrates lack arthroseries glycolipids, which contain the conserved invertebrate-specific core tetrasaccharide GalNAcβ1-4GlcNAcβ1-3Manβ1-4Glc that is synthesized by the BRE pathway. Although the β-linked galactose important for Cry5B binding is not present in this core sequence, our unpublished data indicate that the intact receptor is, by three orders of magnitude, a better competitive inhibitor than β-methylgalactoside. Thus, higher-order structure is likely important for binding. We hypothesize that three-domain Bt Cry toxins evolved to at least partly recognize the core arthroseries tetrasaccharide and thus target the invertebrates, nematodes, and insects that synthesize these molecules.

The high degree of conservation between glycolipids present in *C. elegans* and in the human parasitic nematodes *Ascaris suum* and *Onchocerca volvolus*, which are phylogenetically divergent from *C. elegans*, suggests that most, if not all, nematodes will be susceptible to Cry5B toxin. All the nematodes we have tested to date are susceptible to Cry5B, and the one animal parasite we have tested has been shown to succumb to both Cry5B and Cry14A (*5*). Given these data and evidence that Cry5B and Cry14A toxin use an invertebrate-type glycolipid as their receptor, these Cry proteins hold great promise for one day safely targeting nematode pests of animals and plants.

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Supporting Online Material

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Lymphotoxin-Mediated Regulation of γδ Cell Differentiation by αβ T Cell Progenitors

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The thymus gives rise to two T cell lineages, αβ and γδ, that are thought to develop independently of one another. Hence, double positive (DP) thymocytes expressing CD4 and CD8 coreceptors are usually viewed simply as progenitors of $CD4^+$ and $CD8^+$ αβ T cells. Instead we report that DP cells regulate the differentiation of early thymocyte progenitors and γδ cells, by a mechanism dependent on the transcription factor RORγt, and the lymphotoxin (LT) β receptor (LTβR). This finding provokes a revised view of the thymus, in which lymphoid tissue induction-type processes coordinate the developmental and functional integration of the two T cell lineages.

Cell-mediated immunity involves T cell receptor (TCR) $\alpha\beta^+$ cells, which recognize antigenic peptides presented by major histocompatibility complex (MHC) proteins, and unconventional, non–MHC-restricted T cells, of which $TCR\gamma\delta^+$ cells are the prototype. There is increasing evidence that αβ and γδ T cells are functionally integrated, but their relatedness and how they may "cross-talk" are incompletely understood (*1*). To clarify this situation, we identified a "γδ-biased" gene profile (*2*). Unexpectedly, full expression of this profile by $TCR\gamma\delta^+$ thymocytes depended in trans on $CD4^+CD8^+$ (DP) cells, which are late-stage αβ T cell progenitors that form the most abundant thymocyte subset (Fig. 1A). Reflecting this situation, γδ cell function is altered in $TCR\beta^{-/-}$ mice that lack normal DPs (*2*).

We hypothesized that DP cells might exert their effects directly on maturing γδ T

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