A Role for CD81 in Early T Cell Development

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Early stages of T cell development are thought to include a series of coordinated interactions between thymocytes and other cells of the thymus. A monoclonal antibody specific for mouse CD81 was identified that blocked the appearance of αβ but not γδ T cells in fetal organ cultures initiated with day 14.5 thymus lobes. In reaggregation cultures with CD81-transfected fibroblasts, $CD4^-CD8^-$ thymocytes differentiated into $CD4^+CD8^+$ T cells. Thus, interactions between immature thymocytes and stromal cells expressing CD81 are required and may be sufficient to induce early events associated with T cell development.

Hematopoietic stem cells colonizing the thymus are eventually committed to express either γδ or αβ T cell antigen receptors (TCRs) (*1*). Immature thymocytes present in the outer cortex are $CD4^-CD8^-TCR^-$. These precursors mature into $CD4^+CD8^+TCR^{lo}$ cells, which are then subjected to positive and negative selection events resulting in the emergence of single-positive $CD4^+$ or $CD8^+$ T cells in the thymic medulla. Although thymic stromal cells appear to be necessary for the transition of immature thymocytes to the $CD4^+CD8^+$ stage, limited information is available on the stromal cell surface molecules involved in this process (*2*, *3*). We generated monoclonal antibodies (mAbs) to cell surface determinants expressed by the PAM 212 epithelial cell line and identified one mAb (2F7) that abrogated the appearance of $CD4^+CD8^+$ thymocytes bearing TCRαβ in fetal thymus organ cultures (FTOCs). These cultures were performed with thymus lobes isolated at day 14.5 of gestation, a developmental stage when only $CD4^-CD8^-$ cells can be detected (*4*, *5*). After 7 days, fetal thymus lobes cultured in media alone (Fig. 1A) or in the presence of control mAb 1F3 (*6*) contained about 80% $CD4^+CD8^+$ cells, as evaluated by fluorescence-activated cell sorting (FACS) analyses. In marked contrast, FTOCs in the presence of mAb 2F7 had less than 0.5% $CD4^+CD8^+$ cells (Fig. 1B). This strong inhibitory effect of mAb 2F7 affected mainly αβ T cell development, because an increase in the proportion of γδ T cells was observed in mAb 2F7–treated FTOCs (Fig. 1B). The increased percentage of γδ T cells reflects the absence of αβ T cells. Most of the cells in the mAb 2F7–treated cultures remained $CD4^-CD8^-CD25^+$, a characteristic of early precursor T cells (Fig. 1C). The total number of thymocytes recovered from mAb 2F7–treated cultures decreased dramatically, suggesting a lack of proliferation by $CD4^-CD8^-CD25^+$ thymocytes (Fig. 1D). As expected, very few αβ T cells could be recovered from mAb 2F7–treated cultures, whereas the absolute number of γδ T cells was only slightly reduced compared with control cultures (Fig. 1D). T cell development proceeded normally after removal of mAb 2F7 from day 5 FTOCs, indicating that cytotoxicity was not a likely explanation for our observations (*6*). Thymocytes isolated from disrupted day 14.5 thymus did not react with mAb 2F7 as judged from FACS analyses (*6*). Collectively, the FTOC data suggest that the molecule recognized by mAb 2F7 is expressed by thymic stromal cells and is therefore critical for the generation of $CD4^+CD8^+$ thymocytes. Moreover, mAb 2F7 prevented the cellular proliferation usually associated with the developmental transition of thymocytes from the $CD4^-CD8^-$ stage to the $CD4^+CD8^+$ stage (*7*, *8*).

The molecular size of the mAb 2F7 ligand was determined after surface iodination of PAM 212 cells and immunoprecipitation of cell lysates in the presence of mAb 2F7 and protein A beads. Immune complexes were collected and subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE). An autoradiograph of the resulting gel revealed a major band of an apparent molecular size of 25 kD under reducing conditions (Fig. 2A).

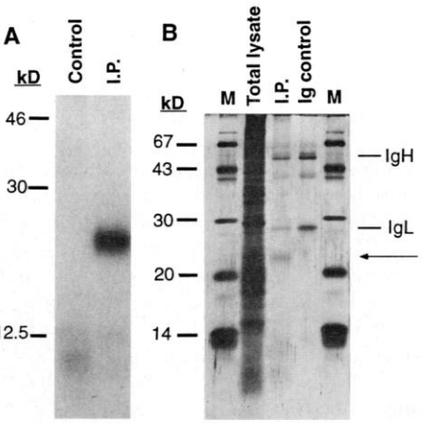

Fig. 2. Identification of the TM4 protein CD81 as the ligand for mAb 2F7 (*27*). (**A**) Monoclonal antibody 2F7 precipitates a unique 25-kD protein at the cell surface of PAM cells. (**B**) The 25-kD protein recognized by mAb 2F7 could be purified after large-scale immunoprecipitation, as evaluated by SDS-PAGE and silver stain. The arrow indicates the position of the 25-kD protein. Sequence analysis of a tryptic peptide from the 25-kD protein yielded the sequence QFYDQALQQAVMDDD-ANNA (mouse CD81 residues 125 to 143). I.P., immunoprecipitation; Ig, immunoglobulin. Abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; F, Phe; L, Leu; M, Met; N, Asn; Q, Gln; V, Val; and Y, Tyr.

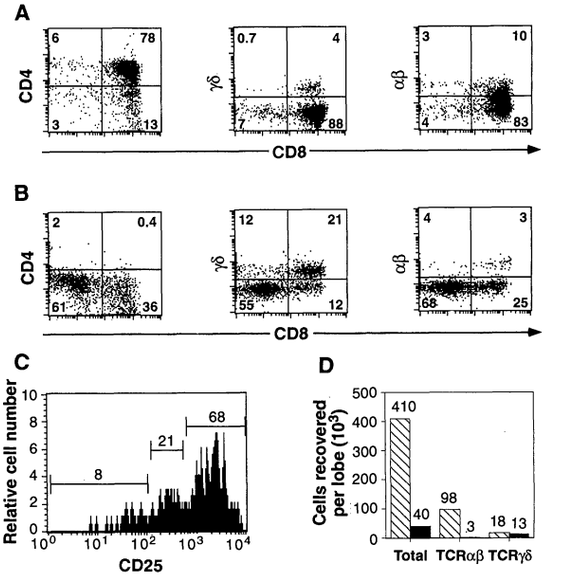

Fig. 1. Monoclonal antibody 2F7 blocks the development of αβ but not γδ T cells (*26*). Fetal thymus lobes at 14.5 days of gestation were cultured for 7 days in FTOC in (**A**) media alone or (**B**) in the presence of mAb 2F7 (25 μg/ml). Thymocytes were recovered from FTOCs and stained with FITC–anti-αβ TCR (mAb H57-597) or FITC–anti-γδ TCR (mAb GL3), phycoerythrin–anti-CD4 (PharMingen), and Red 613–anti-CD8 (Life Technologies) and analyzed by FACS. (**C**) CD25 expression on $CD3^-$ cells obtained after culture for 7 days in the presence of mAb 2F7. Thymocytes were recovered from FTOCs and stained with biotin–anti-CD25 (PharMingen) followed by phycoerythin-streptavidin (Biomeda), FITC–anti-CD3 (mAb 500 A2), and Red 613–anti-CD8. Percentages of CD25 negative, dim, and bright cells are marked. (**D**) Absolute number of cells per thymus lobe recovered after 7 days of culture in the presence of either media alone (striped bars) or mAb 2F7 (black bars). The data are representative of 27 independent experiments.

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The ligand recognized by mAb 2F7 was obtained in quantities sufficient for sequence determination (Fig. 2B). The NH_2-terminal sequence of a tryptic fragment unambiguously identified the 25-kD protein as the murine equivalent to CD81, also known as TAPA-1 (9). CD81 is a member of the transmembrane 4 integral membrane protein family; it has broad tissue distribution but no identified function (*9–12*). Immunohistologic staining of day 14.5 thymus with mAb 2F7 revealed that CD81 was expressed predominantly in the subcapsular region by cells that formed distinguishable clusters (Fig. 3).

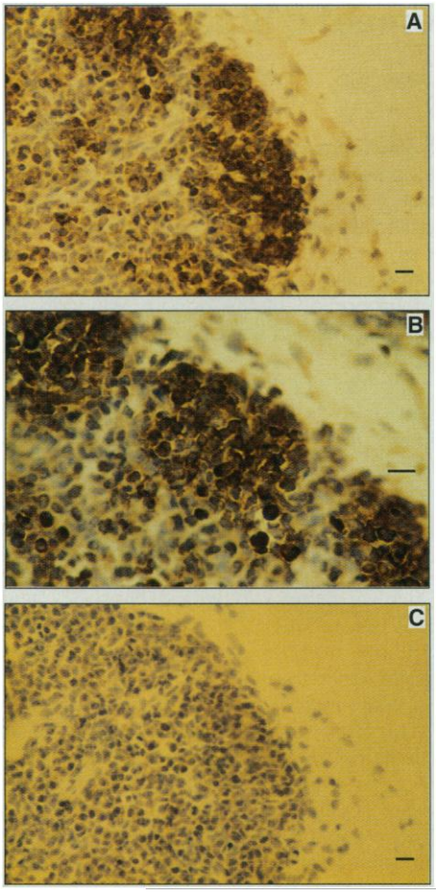

Fig. 3. Localization of CD81$^+$ cells in the fetal thymus (*28*). (**A**) Staining reveals that CD81$^+$ cells are expressed predominantly in the subcapsular region and less frequently in the more central region of the fetal thymus. (**B**) Higher magnification of CD81$^+$ cells localized to the cortical region. (**C**) Control staining performed in the absence of mAb 2F7.

A complete gene for mouse CD81 was obtained by reverse transcriptase–polymerase chain reaction (RT-PCR) with total RNA isolated from PAM 212 cells. Transfected Chinese hamster ovary (CHO) cells expressing CD81 were isolated by FACS with mAb 2F7 as a probe (Fig. 4A). Reaggregation cultures (*5*) consisting of CD4$^-$CD8$^-$ thymocytes and CD81$^+$ CHO cells were used to evaluate directly the contribution of CD81 in early T cell development. In these cultures, most CD4$^-$CD8$^-$ thymocytes transited to a CD4$^+$CD8$^+$ phenotype (Fig. 4C). In contrast, CD4$^+$CD8$^+$ thymocytes were not observed in reaggregation cultures with untransfected CHO cells (Fig. 4B). These observations established that CD81 represents a stromal cell surface protein that is required for early T cell development.

The tissue distribution, molecular size, and function of CD81 suggest that it is not identical with any of the previously defined molecules that function in T cell development. A complex called the pre-TCR, which is composed of a TCR β chain and a glycoprotein, pTα, has been identified at the surface of precursor T cells (*13, 14*). Several lines of evidence suggest a role for the pre-TCR in allelic exclusion at the TCR β locus as well as in the observed proliferation of CD4$^-$CD8$^-$ cells as they differentiate into CD4$^+$CD8$^+$ thymocytes (*7, 8, 15, 16*). The similarities between the events promoted by CD81 and those promoted by the pre-TCR place them in the same functional category (*13, 14, 17, 18*). Both molecules affect T cells at a similar stage of development. Moreover, the development of αβ, but not γδ, T cells is severely blocked under conditions in which the function of these molecules is disrupted (*17*), as shown here for CD81. One possibility is that the pre-TCR expressed by precursor T cells and CD81 present on stromal cells form a functionally relevant receptor-ligand pair. Indeed, previous studies have identified a requirement for stromal cells in the CD4$^-$CD8$^-$ to CD4$^+$CD8$^+$ thymocyte transition (*5*). Furthermore, cross-linking experiments have demonstrated that the pre-TCR associates with CD3 signaling components in a functional manner (*18, 19*). The idea that an extracellular ligand is needed for pre-TCR function is consistent with these observations. An alternative possibility is that precursor T cells need independent signals from the pre-TCR and CD81 to reach the CD4$^+$CD8$^+$ stage. Thus, it may be noteworthy that functional CD3 complexes can be expressed at the surface of immature thymocytes in the apparent absence of the pre-TCR (*20*). Although the exact mechanism by which CD81 and its putative thymocyte ligand interact remains to be clarified, our results add to the molecular foundation needed to elucidate the cell-cell interactions that mediate early events in T cell development.

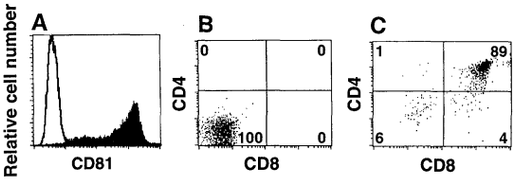

Fig. 4. CD81-transfected cells allow progression of CD4$^-$CD8$^-$ precursor T cells to the CD4$^+$CD8$^+$ developmental stage (*29*). (**A**) Surface staining with mAb 2F7 of CHO-K1 cells before and after transfection with a CD81 cDNA. (**B** and **C**) CD4 and CD8 staining of thymocytes recovered from reaggregation cultures with untransfected CHO-K1 cells (B) or with CD81$^+$ CHO-K1 cells (C). Results are representative of six experiments.

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26. The generation of mAb 2F7 will be described in detail elsewhere (*6*). Briefly, an Armenian hamster was immunized with the PAM epithelial cell line and spleen cells were fused with SP2/0 myeloma cells. Hybridomas secreting mAb reactive with PAM cells were isolated and cloned for further study. FTOC was performed as described (*21*). Briefly, fetal thymus lobes were removed from embryos at day 14.5 of gestation and cultured on polycarbonate filters supported on surgical Gelfoam (Upjohn, Kalamazoo, MI) in 5 ml of complete Dulbecco's minimum essential medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C. After 7 days of culture in medium alone or with mAb (25 μg/ml), the lobes were disrupted and the live cells were counted and then stained with the appropriate conjugated antibodies followed by FACS analysis.
27. Cell surface proteins were iodinated as described (*22*). PAM cells were incubated in lysis buffer con-

sisting of 50 mM tris (pH 8.0), 1% NP-40, 6 mM CHAPS, 150 mM NaCl, 5 mM EDTA, 0.5 mM Pefabloc SC (Boehringer Mannheim), and aprotinin (50 μg/ml; Sigma). Cell lysates were precleared with protein G–Sepharose (Sigma) and incubated with mAb 2F7 at a final concentration of 0.5 μg/ml for 6 hours at 4°C. Protein G–Sepharose was added to a final concentration of 1 μl/ml and immune complexes were allowed to form overnight at 4°C. Immune complexes were collected by centrifugation, and the pellets were washed 20 times with 1 ml of lysis buffer and resuspended in SDS-PAGE buffer containing dithiothreitol. After incubation at 100°C for 5 min, the supernatants were transferred to new tubes and kept at −20°C for further analysis. Standard SDS-PAGE was performed with 10% to 12.5% polyacrylamide gels. When required, proteins were silver-stained with the Silver Stain Plus system (Bio-Rad). For sequence determination, the mAb 2F7 ligand was immunoprecipitated from PAM cells (2×10^{10}), resolved by SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (Millipore). The electroblotted 25-kD protein was excised after visualization with Amido black 10B (Bio-Rad). In situ digestion with trypsin was performed as described previously (*23*). Briefly, the excised band was treated with polyvinylpyrrolidone to prevent binding of the enzyme to the membrane. Digestion with trypsin {1 μg of 30 μl of 0.1 M TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 8.0)]} was allowed to proceed overnight. The supernatant was then fractionated by reversed-phase high-performance liquid chromatography. Fractions were collected manually based on absorbance at 210 nm. A fraction corresponding to a symmetrical peak was subjected to chemical sequence analysis on an ABI 470A protein sequencer (Applied Biosystems, Foster City, CA), and a unique sequence was obtained. The sequence was compared to other known protein sequences with the BLAST program (*24*).

28. Paraformaldehyde-fixed and polyester wax–embedded (*25*) day 14.5 whole embryo sections (10 μm) were hydrated in ethanol, soaked in 0.1 M citrate buffer (pH 6.0), and subjected to microwave treatment for 2 min on the highest setting (Radarange 1000W; Amana, IA). This treatment substantially improved the staining intensity obtained with mAb 2F7. The endogenous peroxidase activity was blocked, and the sections were incubated for 30 min in 2% FBS. The sections were then stained for 2 hours with biotinylated mAb 2F7, adjusted to 10 μg/ml in 1% FBS, and washed. Binding was visualized with streptavidin-labeled peroxidase (Jackson ImmunoResearch), followed by incubation in metal-enhanced diaminobenzidine (Pierce). No staining was observed when mAb 2F7 was omitted from the procedure. The sections were counterstained with hematoxylin, dehydrated in ethanol, cleared with Hemo-De (Fisher Scientific, Pittsburgh, PA), and mounted with DPX reagent (British drug house).
29. A complete murine CD81 complementary DNA (cDNA) was encoded by RT-PCR with oligonucleotide primers 5′-CGGAATTCATGGGGGTGGAGGGCTG-3′ and 5′-CGGAATTCTCAGTACACGGAGCTGTTC-3′. These primers were designed from the published murine CD81 gene sequence (*9*) and contained Eco RI restriction sites for cloning purposes. The complete CD81 cDNA was introduced into the eukaryotic expression plasmid pcDNA3 (Invitrogen) at the Eco RI site in the correct orientation for expression. We established stable CD81-expressing cells with Lipofectamine-mediated (Life Technologies) transfection of CHO-K1 cells followed by G418 selection. CD81 transfectants were selected by FACS after sequential incubations with biotinylated mAb 2F7 and fluorescein isothiocyanate (FITC)–streptavidin (Biomeda, Foster City, CA). Reaggregation cultures were performed as described (*5*). In brief, thymocytes (2×10^5) isolated from day 14.5 fetal thymus lobes were mixed with untransfected or CD81$^+$ CHO-K1 cells (1×10^5) centrifuged at 2000 rpm for 2 min in a microcentrifuge (Eppendorf), and the pellet resuspended in complete DMEM–10% FBS (4 μl). Before reaggregation, thymus cell preparations typically contained 98% CD25$^+$ cells and 2% CD81$^+$ cells, as evaluated by FACS analyses. Cells were deposited in 1-μl drops on the surface of polycarbonate filters supported with surgical Gelfoam (Upjohn) in complete DMEM–10% FBS (5 ml) and cultured for 5 days. After culture, cell pellets were disaggregated by resuspension in complete DMEM–10% FBS and stained with phycoerythrin antibody to CD4 (anti-CD4) and Red 613 antibody to CD8 before FACS analysis.

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Effect of Polymorphism in the *Drosophila* Regulatory Gene *Ultrabithorax* on Homeotic Stability

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Development is buffered against unpredictable environmental and genetic effects. Here, a molecular genetic analysis of one type of developmental homeostasis, the establishment of thoracic segmental identity under the control of the *Ultrabithorax* (*Ubx*) gene in *Drosophila melanogaster*, is presented. Flies were artificially selected for differential sensitivity to the induction of bithorax phenocopies by ether vapor. The experiments demonstrated that increased sensitivity to ether correlated with a loss of expression of UBX in the third thoracic imaginal discs and that a significant proportion of the genetic variation for transcriptional stability can be attributed to polymorphism in the *Ubx* gene.

The specification of segmental identity in *Drosophila* depends on the coordination of complex expression patterns of homeotic genes in the Antennapedia and Bithorax complexes (*1*). This specification must also be a highly stabilized process, because morphological uniformity is produced despite environmental and genetic variation. However, as was shown by Gloor (*2*), the specification of segmental identity can nevertheless be disrupted by exposure of early embryos to ether vapor, which induces bithorax phenocopies that resemble homeotic transformations caused by mutations in the regulatory regions of the *Ubx* gene (*3*). Waddington later showed, by selecting populations that exhibit increased or decreased phenocopy frequencies, that genetic variation affects this process (*4*).

Starting with an outbred population of flies (*5*), we performed a selection experiment similar to that described by Waddington. The Ives strain is free from inversions and has been deliberately maintained with a high degree of heterozygosity (*6*). Embryos were collected at room temperature over a 1-hour period, and 2.5 hours later (that is, 3.0 ± 0.5 hours after eggs were laid) they were exposed to ether vapor for 10 min. More than 20 groups of about 400 embryos were treated each generation; upon emergence, the adults were scored for bithorax phenocopies. More than 1500 adults showing identity transformations of the third thoracic segment (T3), ranging from ectopic sternopleurae to near-complete replacement of halteres by wings (Fig. 1A), were selected for the next generation. Flies exhibiting such phenocopies were selected for eight generations. A steady increase in phenocopy frequency was observed in each generation (Fig. 2A, experiment 1), accumulating from 13% in the starting population to a plateau of 45%. Similar results were obtained in repetitions of the experiment 3 months later (Fig. 2A, experiment 2) and 2 years later (*7*). By contrast, the frequency of bithorax phenocopies dropped steadily when flies were selected for resistance to ether treatment (Fig. 2A, experiment 3) by breeding only from nontransformed flies. The results show that genetic variation exists for the propensity to exhibit ether-induced bithorax phenocopies in the Ives strain of *D. melanogaster* (*8*).

Several observations implicate the *Ubx* gene in the response to selection. (i) Many loss-of-function mutations in *Ubx* produce bithorax transformations similar to the ether-induced phenocopies (*9*). (ii) Flies heterozygous for mutations in genes that regulate the activation and maintenance of *Ubx* expression (including *hunchback* and *Polycomb*) exhibit altered bithorax phenocopy frequencies (*10*). (iii) Ether induced loss of UBX expression in patches within

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