counted. Of the 98 cuticles examined for Fig. 2F, 44 were completely open dorsally (like the example in Fig. 2F), whereas 54 were only partially open dorsally (like Fig. 2C). This distribution corresponds statistically to a 1:1 ratio, consistent with half of the mutant *kay*^{2} embryos having received a mutant DJun allele and the other half having received a balancer chromosome, wild type for *DJun*. For Fig. 2G, 311 cuticles were examined; 151 were completely open dorsally (like Fig. 2G), and 160 were only partially dorsal open (like Fig. 2E). The distribution statistically conforms to a 1:1 ratio, as expected from half of the mutant embryos having received a mutant *bsk*^{1} allele, and half a balancer chromosome, wild type for *bsk*. All mutant *kay*^{2} or *kay*^{1}/*kay*^{2} cuticles examined were always only partially open dorsally. For Fig. 2H, 47 embryos were examined: 21 were three-quarters or more open dorsally (like Fig. 2H), and 26 were half or less open dorsally [see (*8*) for examples]. This distribution also corresponds statistically to a 1:1 ratio, as expected from half of the mutant *bsk*^{1}/*Df(2L)flp*^{147E} having received a mutant *kay*^{2} allele, and half a balancer chromosome. In all tests here and in (*24*), significance was assayed by a chi-squared test and was found in all cases to be $P < 0.05$.

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Requirement for CD44 in Activated T Cell Extravasation into an Inflammatory Site

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Leukocytes extravasate from the blood into inflammatory sites through complementary ligand interactions between leukocytes and endothelial cells. Activation of T cells increases their binding to hyaluronate (HA) and enables CD44-mediated primary adhesion (rolling). This rolling could be induced in vivo in murine $V_\beta8^+$ T cells in response to specific superantigen stimulation; it was initially found in lymph nodes, then in peripheral blood, and finally within the peritoneum, the original inflamed site. The migration of $V_\beta8^+$ cells into the peritoneal cavity was dependent on CD44 and HA, as shown by inhibition studies. Thus, CD44-HA interactions can target lymphocytes to specific extralymphoid effector sites.

The compartmentalization, organization, and function of the immune system has engendered the development of specialized trafficking and recirculation patterns for subsets of lymphocytes that differ from those of other leukocytes. In particular, tissue-specific migration pathways of effector and memory lymphocyte subsets have been shown to be distinct and in part attributable to adhesion receptors they express (*1–4*). Since the inception of the model of sequential receptor engagement leading to leukocyte extravasation, only the selectin and integrin families of proteins have been implicated in primary (rolling) and secondary (firm) adhesion (*5*, *6*). The principal ligand for CD44, a marker of effector and memory lymphocytes, is HA (*7*), on which activated T cells can roll under physiologic shear stress conditions (*8*). In vivo activation of T cells and direct stimulation through the T cell receptor (TCR) results in CD44 capable of binding HA (*9*, *10*). Together, these findings suggest that CD44-HA interactions may be important for lymphocyte extravasation at inflammatory sites. We investigated whether T cell sensitization to antigen, occurring primarily within secondary lymphoid tissues, resulted in the induction of the "activated" HA-binding form of CD44 on antigen-specific cells; whether the stimulated cells bearing activated CD44 were mobilized into the peripheral circulation; and whether the activated CD44 on these cells then facilitated their extravasation into an inflammatory site.

Staphylococcal enterotoxin B (SEB) preferentially stimulates $V_\beta8^+$ T cells (*11*), which initially accumulate in the periphery and then are lost by 2 to 4 days after injection of SEB (*12*, *13*). We used intraperitoneal (ip) SEB injection of mice as an approach to obtain in vivo activated T cells, which we then examined by laminar flow and flow cytofluorometric analysis during the course of the response. For direct comparison of $V_\beta8$ composition and rolling activity, mesenteric lymph nodes (MLNs), peripheral blood, and peritoneal exudate leukocytes (PELs) were harvested at 4-hour intervals. The proportion of $V_\beta8^+$ cells in draining MLNs remained stable (at 15%) throughout this time course (Fig. 1A). Almost all $V_\beta8^+$ cells disappeared from peripheral blood by 4 hours after SEB injection, consistent with previous reports of sequestration of antigen-specific lymphocytes in secondary lymphoid organs at early time points after antigen injection (*14–16*). This drop was followed by a rapid rise of $V_\beta8^+$ T cells over the next 4 to 8 hours, followed by a gradual decline. In PELs, the percentage of $V_\beta8^+$ cells also initially decreased by 4 hours but then rose over the next 12 hours. The beginning of an accumulation of $V_\beta8^+$ cells in the peritoneal cavity was coincident with the peak of $V_\beta8^+$ cells in peripheral blood (Fig. 1A).

The HA-binding population in draining nodes after SEB injection was examined by flow cytometric analysis. After SEB treatment, 4% of all MLN lymphocytes bound fluoresceinated HA (Fl-HA), whereas binding was barely detectable in cells from control animals (Fig. 1B). This binding was inhibited by preincubation with monoclonal antibody (mAb) KM81, which inhibits the binding of activated CD44 to soluble HA (*17*). Of the total $V_\beta8^+$ lymph node population, 8.5% were $CD44^+$ and bound HA, whereas 17% of the $CD69^+$ and 28% of the lymphoblast populations were $CD44^+$ and bound HA. No HA binding was seen in $CD69^-$ cells, which suggested that CD44-dependent HA binding is a feature of activated but not resting cells. Thus, in vivo stimulation with SEB resulted in the activation of the specific $V_\beta8^+$ T cell subset within draining lymph nodes, which increased its HA binding and the potential for CD44-mediated primary adhesion.

The pattern of appearance of cells able to engage in CD44- and HA-dependent rolling interactions also showed sequential appearance in each anatomic compartment (Fig. 1C). MLN cells showed an early increase in rolling, which appeared 4 hours later in peripheral blood lymphocytes and not until 8 hours later in the peritoneal cavity. Although the timing of the appearance of rolling activity varied between experiments, the sequential appearance of both $V_\beta8$ cells and rolling activity in the three compartments in all experiments was maintained. Thus, the accumulation of rolling cells in peripheral blood and peritoneum paralleled the appearance of $V_\beta8^+$ lymphocytes in these sites, and it was consistent with the origination of CD44-mediated rolling cells within lymph nodes, with subsequent release into peripheral blood followed by extravasation into the inflamed peritoneum.

Cell populations were fractionated to identify which cells rolled after SEB injection (*18*). In all three compartments, the preponderance of the rolling activity was found in T cells, particularly the $V_\beta8^+$ sub-

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set, but not in T cell–depleted or $V_\beta8^-$ populations (Fig. 2). Rolling activity in MLN cells was absent in the similarly selected and well represented (~12%) control $V_\beta14^+$ population. Primary adhesion was effectively blocked by KM81, and depletion of HA-binding cells also resulted in the removal of rolling activity. No rolling activity was seen in $V_\beta8$ cells from animals injected with phosphate-buffered saline (PBS) only. Thus, in each compartment, the activated $V_\beta8^+$ cells expressed the CD44- and HA-dependent primary adhesion activity.

To address the role of CD44 in the in vivo trafficking of activated $V_\beta8^+$ T cells from the blood into the peritoneum, we injected mice ip with SEB and then intravenously (iv) with KM81 or isotype-matched control antibodies 4 hours later (Fig. 3). In vivo, KM81 inhibited the influx of $V_\beta8^+$ T cells into the peritoneum, whereas control antibodies did not have this effect. This was not attributable to the loss of these cells from the animal, because both circulating and peripheral lymphoid (lymph nodes and spleen) $V_\beta8$ cells were not diminished by this treatment (*19*). However, in four separate experiments, KM81 consistently inhibited cell migration with effectiveness ranging from 70 to 90%. The total number of intraperitoneal cells (consisting primarily of monocytes, macrophages, and neutrophils) was not altered by these antibody treatments, indicating no effect on trafficking of these other populations. Thus, the trafficking of stimulated antigen-specific $V_\beta8^+$ T cells into the inflamed peritoneal site appears to be dependent on CD44.

To directly characterize the migration of cells activated after SEB ip injection, we conducted short-term homing experiments (*20*). Cells were isolated from draining MLNs of mice treated with SEB 20 hours earlier. These cells were fluorescently labeled and injected iv into recipient mice that had received SEB ip 20 hours earlier to create an inflamed site, or into PBS-injected control mice. Total lymph node cells were injected alone, together with antibody, or after depletion of HA-binding cells. Cells in the recipient peritoneal cavities were collected and analyzed for green [CFDA (*20*)] fluorescence 90 min after injection. SEB-treated mice, but not control mice, receiving cells from MLNs of SEB-injected donors showed an influx of cells into the peritoneal cavities (Fig. 4A). Cells from untreated donor animals showed no substantial migration into SEB-inflamed peritoneum. Entry of cells into the peritoneum was specifically blocked by coadministration of KM81 mAb, whereas the isotype-matched mAbs MEL-14 and anti–H-2 had no significant effect. MEL-14 did, however, inhibit lymph node homing in the same experiments (*19*). Removal of the HA-binding cells also abrogated homing, suggesting that HA is an in vivo ligand.

Because cells that entered the peritoneum after SEB injection retained the ability to undergo CD44-dependent rolling, we examined whether this PEL population was capable of reentry into the inflamed site. Donor

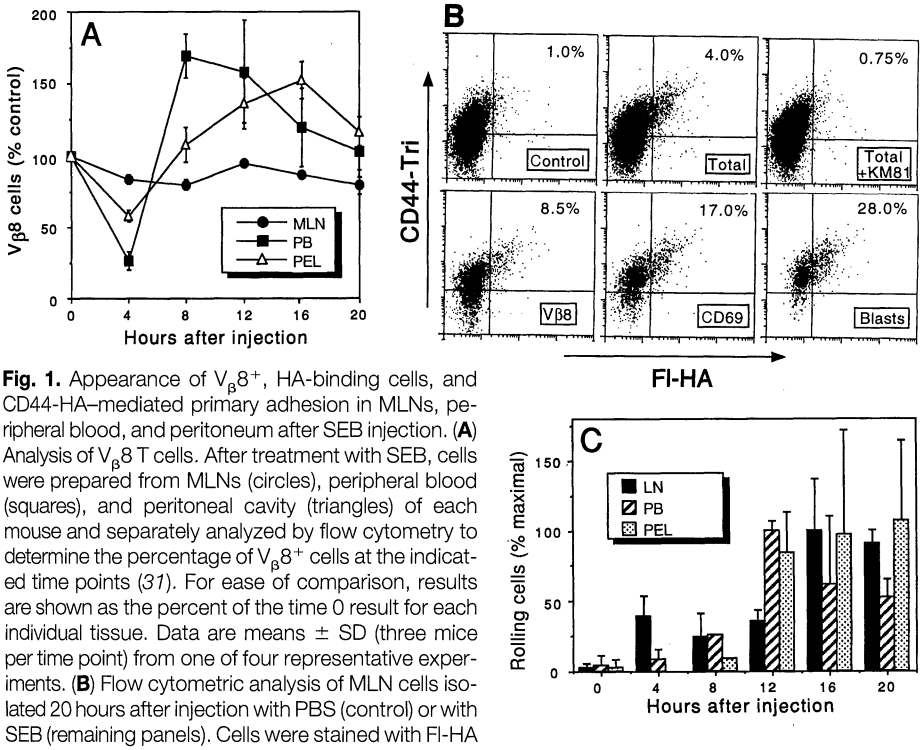

Fig. 1. Appearance of $V_\beta8^+$, HA-binding cells, and CD44-HA–mediated primary adhesion in MLNs, peripheral blood, and peritoneum after SEB injection. (**A**) Analysis of $V_\beta8$ T cells. After treatment with SEB, cells were prepared from MLNs (circles), peripheral blood (squares), and peritoneal cavity (triangles) of each mouse and separately analyzed by flow cytometry to determine the percentage of $V_\beta8^+$ cells at the indicated time points (*31*). For ease of comparison, results are shown as the percent of the time 0 result for each individual tissue. Data are means ± SD (three mice per time point) from one of four representative experiments. (**B**) Flow cytometric analysis of MLN cells isolated 20 hours after injection with PBS (control) or with SEB (remaining panels). Cells were stained with Fl-HA (*32*), anti-CD44 (IM7) + GαRIg-Tricolor (Cappel), and either anti-$V_\beta8$-PE or anti–CD69-PE. For analysis of anti–CD44-Tricolor versus Fl-HA staining, samples were gated on total lymphocytes or, to analyze activated populations, $V_\beta8^+$ cells, $CD69^+$ cells, or blasts (forward versus side scatter gating), as indicated. Blocking of Fl-HA staining of total lymphocytes by addition of KM81 mAb for 5 min before addition of Fl-HA is also shown. (**C**) Rolling interactions of cells from all three sites were determined at the indicated time points. Results are shown as percent of maximal rolling for each tissue (maximal values: MLN cells = 15, peripheral blood = 4, PELs = 6 cells rolling). Physiological flow conditions were produced with a parallel-plate flow chamber, as described (*8, 33*). Data are from the same representative experiment shown in Fig. 1A (three mice per time point).

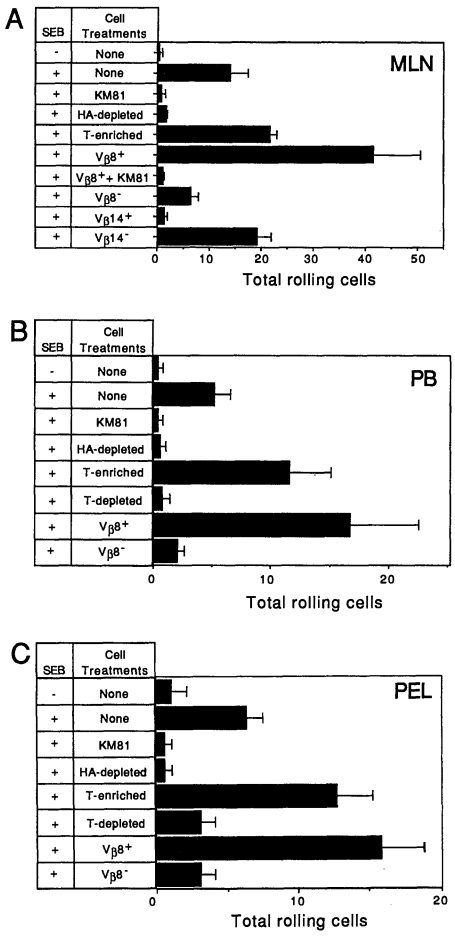

Fig. 2. Analysis of CD44-HA–mediated primary adhesion of lymphocytes isolated from MLNs, peripheral blood, and peritoneal cavity after SEB injection. Cells were isolated from mice injected ip 20 hours earlier with PBS (–) or SEB (+) and analyzed for rolling in the parallel-plate flow chamber. Cells were used without treatment (None), in the presence of KM81 mAb, or after fractionation or depletion of cell populations, as indicated (*18*). All data are means ± SEM of three experiments. (**A**) MLN cells; (**B**) peripheral blood cells; (**C**) peritoneal exudate cells.

PELs entered recipient peritoneal cavities in significant numbers, and KM81, but not control mAbs, blocked this migration (Fig. 4B). Depletion of HA-binding cells or T lymphocytes removed the cells that could traffic to the recipient inflamed site, although T cells constituted only ~20% of PELs under these conditions. The fluorescent cells that migrated into the peritoneum had a forward and side light scatter profile characteristic of lymphoblasts (*19*). Thus, upon direct transfer, SEB-activated peritoneal T cells migrate into an inflamed peritoneal site in an HA- and CD44-dependent manner.

We next examined the nature of the ligand because CD44 has been described to bind other molecules (*7, 21*). Donor lymph node cells were first incubated with Fab fragments of either KM81 (HA-blocking) or KM703 (non–HA-blocking) before injection (Fig. 4C). KM81 inhibited entry of donor cells into the peritoneum, whereas KM703 had no effect, although surface staining of the two antibodies was equivalent (*19*). Thus, the determinants on CD44 involved were those associated with its HA-binding function. Homing to the peritoneum could also be inhibited directly by infusion iv with HA, but not with the similar disaccharide polymer chondroitin sulfate A (CSA) (Fig. 4C), even at a 10-fold molar excess (*19*). Another carbohydrate polymer, yeast mannan, also failed to inhibit the entry of labeled cells into inflamed peritoneal cavities (*19*). In addition, treatment of recipient mice iv with hyaluronidase (*22, 23*) before the infusion of donor cells inhibited peritoneal recruitment; chondroitinase did not have this effect (Fig. 4C). Pretreatment of the donor cells alone with hyaluronidase before injection did not affect homing, which suggested that the hyaluronidase had its effect within the vasculature of the recipient rather than on donor cells (*19*). The results of these various approaches indicate that HA is the operative ligand in this system.

The role in lymphocyte trafficking that we demonstrated for CD44 is distinct from the prior association of CD44 with mechanisms of human lymphocyte homing to secondary lymphoid organs (*24, 25*). The basis for the human observations remains unresolved, and anti-CD44 treatment has been reported not to affect normal lymphocyte recirculation in mice (*26, 27*). Our results add CD44 to the repertoire of adhesion receptors that can be used by leukocytes during extravasation, in this model into a site of peritoneal inflammation. Although our characterization suggests that CD44 contributes in this pathway through primary adhesion, the basis for secondary adhesion remains to be elucidated. Secondary adhesion is potentially mediated by integrins of the α_4 and β_2 families (or both). CD44, widely distributed on most hematopoietic as well as other cell types, has been studied in numerous systems and has many apparent functions, including extracellular matrix binding, lymphocyte homing and activation, lymphopoiesis, and metastasis (*7*). CD44 may have a role in human arthritis (*28*), in a collagen-induced model of murine arthritis (*27, 29*), and in a contact hypersensitivity response (*26*). Thus, it is possible that the basis for the association of normal and transformed cell trafficking with CD44 in these models may be attributable in part to the CD44-HA interaction we described. We have suggested that lymphocyte stimulation would induce activation of CD44 to bind HA, and thus differentiation would culminate in new effector and homing functions; in conjunction with

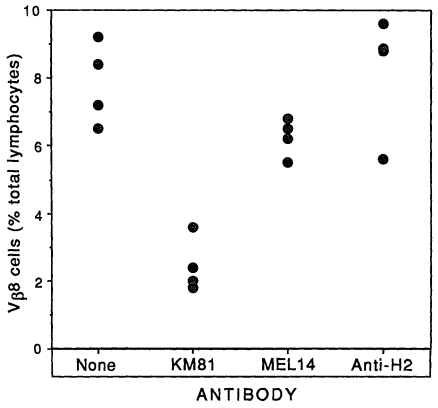

Fig. 3. CD44 mAb KM81 prevents entry of $V_\beta8^+$ T cells into SEB-injected peritoneum. Mice were injected ip with 50 μg of SEB as above. Four hours later, mice were injected iv with 300 μg of KM81 or isotype-matched control mAbs MEL-14 or anti–H-2 (M1/42, rat antibody to mouse pan–H-2) (*34*) in 500 μl of sterile PBS. One group of mice received no antibody (None). 20 hours after the initial SEB injection, PELs were collected by lavage and cells were analyzed by FACS for $V_\beta8^+$ cells. The difference between nonblocked and KM81-treated mice was significant ($P < 0.01$), whereas the difference between nonblocked and MEL-14– or M1/42-treated mice was not ($P > 0.5$). Data are from one representative experiment. The mean total leukocytes (± SD) in the PEL harvest after treatment with SEB ($2.1 \pm 0.5 \times 10^6$), SEB + KM81 ($2.3 \pm 0.4 \times 10^6$), and SEB + MEL-14 ($2.4 \pm 0.8 \times 10^6$) indicated no gross alterations in cellular influx resulting from antibody treatment.

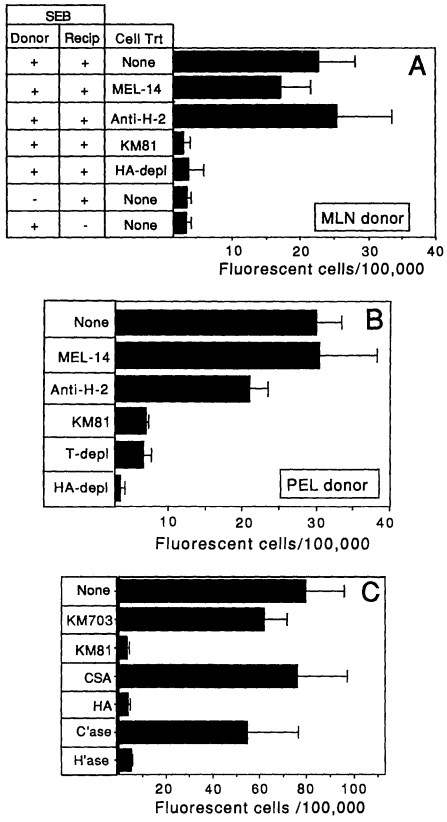

Fig. 4. CD44-dependent short-term homing of activated T cells into an inflamed site. (**A**) CFDA-labeled MLN cells from SEB-treated (+) or untreated (–) donor mice were injected iv into either SEB-treated (+) or PBS-treated (–) recipients. Cells from donor mice were injected alone (None), with KM81 mAb, with isotype-matched control mAbs MEL-14 or anti–H-2, or after depletion of HA-binding cells as in Fig. 2. Cells were recovered from recipient mice by peritoneal lavage and analyzed by FACS for CFDA fluorescence to determine the number of transferred cells present in the peritoneal cavities. Data are shown as the number of fluorescent cells per 100,000 cells analyzed and represent means ± SEM from two or three separate experiments (three mice per group in each experiment; $n \geq 6$). KM81 treatment and HA depletion significantly reduce the number of cells found in peritoneal cavities of SEB-treated recipients ($P < 0.005$), whereas MEL-14 and anti–H-2 treatment do not ($P > 0.3$). (**B**) CFDA-labeled peritoneal exudate cells from SEB-treated donor mice were injected iv into SEB-treated recipients alone (None), with KM81 or control mAbs, or after depletion of T cells or HA-binding cells. Data are from at least two experiments (three mice per group in each experiment; $n \geq 6$). KM81, T cell depletion, and HA depletion all reduced the number of cells found in peritoneal cavities of SEB-treated recipients ($P < 0.005$), whereas MEL-14 and anti-H-2 treatment did not ($P > 0.3$). (**C**) CFDA-labeled MLN cells from SEB-treated mice were injected iv into SEB-treated recipients in HBSS alone (None), after incubation with Fab fragments of KM81 (HA-blocking) or KM703 (non–HA-blocking), or with HA or CSA. Some recipient mice were also treated with hyaluronidase (H'ase) or chondroitinase ABC (C'ase) before infusion of donor cells, as indicated. Data are from two or three separate experiments (three mice per group in each experiment; $n \geq 6$). KM81 Fab treatment of cells and hyaluronidase treatment of animals each significantly reduced the number of fluorescent cells in peritoneal cavities of recipients ($P < 0.05$), whereas KM703 Fab and chondroitinase ABC did not ($P > 0.2$).

HA on endothelium induced by proinflammatory stimuli (30), this ligand pair would participate in the process of extravasation into these sites (8).

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18. M. H. Julius, E. Simpson, L. A. Herzenberg, *Eur. J. Immunol.* **3**, 645 (1973). Enrichment of T lymphocytes from blood, lymph node, and peritoneal samples was done by nylon wool nonadherence. Resulting cell samples contained >80%, >95%, and >80% T cells, respectively, based on flow cytometric analysis with antibody to $TCR_{\alpha\beta}$. T cell depletion of peripheral blood and peritoneal samples was done with anti-Thy1.2 conjugated magnetic beads (Dynal). Cells (5×10^6) were incubated for 20 min with 10^7 beads in 1 ml of RPMI 1640 with 5% fetal bovine serum (FBS) at 4°C on a rocking platform. $Thy1.2^+$ T cells were removed by magnetic separation. After depletion, <1% of the remaining cells were shown to be T cells by flow cytometric analysis. Depletions of $V_\beta8^+$ T cells and HA-binding cells were done with anti-V_β8–biotin or Fl-HA + anti-fluorescein isothiocyanate (FITC)–biotin, respectively, and then with streptavidin-conjugated magnetic beads (Dynal). Depletion of $V_\beta14^+$ T cells was done with anti-V_β14 and GαRIg-conjugated beads (Dynal). Control samples were incubated with streptavidin-conjugated beads alone. For positive selection of $V_\beta8^+$ T cells, cells were incubated with anti-V_β8–biotin in the presence of 10% normal mouse serum, washed, and then incubated with streptavidin-FITC. After washing twice, cells were further incubated with anti-FITC magnetic particles and separated from the total population using a MACS magnetic separation column (Miltenyi Biotec). Fluorescence-activated cell sorting (FACS) analysis of this population showed them to be 85 to 90% $V_\beta8^+$. Positive selection of $V_\beta14$ T cells was done with anti-V_β14, anti-κ–FITC, and anti-FITC particles.
19. H. C. DeGrendele, P. Estess, M. H. Siegelman, data not shown.
20. For short-term homing assays, cells were labeled by resuspending at a concentration of 10^7 cells/ml in Hanks' balanced salt solution (HBSS) containing 2 μM 5- (and 6-)carboxylfluorescein diacetate, succinimidyl ester (CFDA, Molecular Probes), incubating at room temperature for 20 min, then washing twice in HBSS. Recipient BALB/c mice [injected ip 20 hours earlier with SEB (50 μg) or with PBS] were then injected iv with 10^7 labeled cells per mouse in 0.5 ml of HBSS with or without the addition of blocking reagent. Depletions were done as above. Cells treated with whole antibody or Fab fragments were incubated in a saturating concentration of antibody for 15 min on ice and then washed before injection. HA and chondroitin sulfate (Sigma) were added to the cell suspension at the time of injection (final concentration, 0.5 μM). Hyaluronidase (ICN Biochemicals) and chondroitinase ABC (Sigma) treatment was done by injecting 10 U per mouse iv 30 min before donor cell infusion. Ninety minutes after the infusion of labeled cells, the recipient mice were killed and PELs were analyzed for the homing of labeled cells.
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31. BALB/c mice were injected ip with 50 μg of SEB (Sigma) in 500 μl of sterile PBS and killed at 4-hour intervals for analysis. Controls were injected with PBS alone. MLNs were processed into single-cell suspensions and the percentage of $V_\beta8^+$ lymphocytes was determined by flow cytometry using anti-V_β8-phycoerythrin (PE) (Pharmingen). Peripheral blood was collected by cardiac puncture into Alsever's solution and red blood cells were lysed by incubation in 0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM EDTA. PELs were collected by peritoneal lavage using 5 ml of RPMI (37°C) containing 2% FBS and 2 mM EDTA. Staining of PELs was performed as above, except cells were preincubated with anti-CD32/CD16 (clone 2.4G2) and stained in the presence of 10% normal mouse serum to inhibit Fc receptor interactions. Data were collected on a FACScan analytical instrument (Becton Dickinson) and analyzed using Lysis II software.
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Functional Coherence of the Human Y Chromosome

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A systematic search of the nonrecombining region of the human Y chromosome (NRY) identified 12 novel genes or families, 10 with full-length complementary DNA sequences. All 12 genes, and six of eight NRY genes or families previously isolated by less systematic means, fell into two classes. Genes in the first group were expressed in many organs; these housekeeping genes have X homologs that escape X inactivation. The second group, consisting of Y-chromosomal gene families expressed specifically in testes, may account for infertility among men with Y deletions. The coherence of the NRY's gene content contrasts with the apparently haphazard content of most eukaryotic chromosomes.

Functional or developmental themes have rarely been ascribed to whole chromosomes in eukaryotes. Instead, individual chromosomes appear to contain motley assortments of genes with extremely heterogeneous patterns of developmentally regulated expression. We speculated that the human Y chromosome might be a functionally coherent exception, at least in its nonrecombining portion (the NRY), which makes up 95% of its length (*1*). It is known to differ from all other nuclear human chromosomes by the absence of recombination, its presence in males only, its common ancestry and persistent meiotic relationship with the X chromosome, and the tendency of its genes to degenerate during evolution (*2*).

From the 1950s to the present day, many biologists have assumed that the Y chromosome is a functional wasteland, despite the discovery of several NRY genes during this period. Studies of human pedigrees had identified many traits exhibiting autosomal or X-linked inheritance but no convincing cases of Y-linked inheritance (*3*). In 1959, reports of XO females and XXY males established the existence of a sex-determining gene on the human Y chromosome (*4*), but this was perceived as a special case on a generally desolate chromosome. The wasteland model has been revised only during the past decade, when eight NRY transcription

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