Physiological Migration of Hematopoietic Stem and Progenitor Cells

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Physiological Migration of Hematopoietic Stem and Progenitor Cells

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Hematopoietic stem cells (HSCs) reside predominantly in bone marrow, but low numbers of HSCs are also found in peripheral blood. We examined the fate of blood-borne HSCs using genetically marked parabiotic mice, which are surgically conjoined and share a common circulation. Parabionts rapidly established stable, functional cross engraftment of partner-derived HSCs and maintained partner-derived hematopoiesis after surgical separation. Determination of the residence time of injected blood-borne progenitor cells suggests that circulating HSCs/progenitors are cleared quickly from the blood. These data demonstrate that HSCs rapidly and constitutively migrate through the blood and play a physiological role in, at least, the functional reengraftment of unconditioned bone marrow.

Bone marrow (BM) transplantation is a common procedure wherein suspensions of cells are harvested from marrow cavities and intravenously injected into patients or animals whose blood-forming system is compromised by radio- and/or chemotherapy. The donor cells colonize the ablated marrow and reconstitute blood-forming capacity (*1*). It is not clear, a priori, why BM transplantation should work. Why should hematopoietic stem and progenitor cells isolated from resting BM accurately home to hematopoietic BM niches? Is this simply a random event or are the cells taking advantage of a normal physiological process—blood to BM homing—that exists in the absence of BM ablation? To investigate these questions, we examined the behavior of the hematopoietic stem and progenitor cells that normally exist in the blood.

Although most adult HSCs reside in BM and marrow is the principal site of adult hematopoiesis, it has been known for nearly 40 years that hematopoietic precursors are also present in the peripheral blood of normal animals (*2*–*4*). However, whether blood-borne HSCs play a role in BM or extramedullary hematopoiesis has remained unclear. To evaluate the physiological relevance of circulating HSCs, we generated pairs of parabiotic mice, in which partners differed at the CD45 locus (CD45.1 and CD45.2) (*5*–*7*). Cross circulation was established by day 3 after surgical joining, and blood chimerism reached ~50% by days 7 to 10 (Fig. 1). Thereafter, blood chimerism remained stable, with no significant difference between chimerism of CD45.1 and CD45.2 partners (*8*).

To test for engraftment of unconditioned BM by circulating HSCs, we separated established pairs of parabiotic mice for 7, 10, or 22 weeks before analysis of their blood and BM for chimerism. BM was analyzed by flow cytometry for the presence of partner-derived long-term (LT) HSCs, defined by the surface phenotype Thy-1.1^{lo}Lineage^{-}Sca-1^{+}c-Kit^{+} (*9*–*13*), and blood was analyzed for the presence of partner-derived mature blood cells (Fig. 2 and Table 1). After 7 weeks of parabiosis, surgical separation, and an additional 7 weeks as individuals, nine of nine surviving mice from five pairs had chimeric granulocyte compartments (Table 1, experiment 1). Because granulocytes are short lived [life-span ~1 day (*14*)] and maturation of these cells from nonrenewing progenitor cells requires less than 4 weeks (*15*), partner-derived granulocytes present 7 weeks after separation must have been generated from BM engrafting LT-HSCs, which give rise to granulocytes for life, or from short-term HSCs (ST-HSCs), which give rise to granulocytes for only ~6 to 8 weeks (*9*).

Ten or 22 weeks after separation, previously parabiosed mice were killed and analyzed for chimerism of BM LT-HSCs (Fig. 2 and Table 1). In five of five surviving mice from three pairs analyzed after 22 weeks of separation, all mice had partner-derived peripheral blood granulocytes, and all mice had partner-derived BM LT-HSCs in about the same proportions as the granulocytes (r^2 = 0.983) (*16*). In one pair (pair 5), analyzed after 10 weeks of separation, the CD45.1, but not the CD45.2, partner was chimeric for LT-HSCs, indicating that the partner-derived granulocytes observed at 7 weeks in the CD45.2 animal were descendants of engrafting ST-HSCs.

In mice that have received granulocyte colony-stimulating factor (G-CSF) or cyclophosphamide (Cy) plus G-CSF to mobilize HSCs and progenitors into blood, the spleen becomes a major homing site of mobilized cells (*11*, *17*). However, the spleen is only a low-efficiency site of hematopoiesis (*18*), and so, in parabiotic pairs 2 and 3 (Table 1), we splenectomized one partner before parabiosis to attempt to enhance our ability to detect partner-derived marrow LT-HSC engraftment and hematopoiesis (*19*). The splenectomized CD45.1 partner of pair 2 died before analysis, but the surviving CD45.2 partner had high levels of granulocyte and BM LT-HSC chimerism 22 weeks after separation (15% and 20%, respectively). In pair 3, both the splenectomized CD45.2 mouse and its CD45.1 partner exhibited peripheral blood granulocyte chimerism (0.3% and 5.2%, respectively). The relatively high levels of chimerism in partners of splenectomized mice are consistent with previous reports describing increased numbers of circulating progenitors after splenectomy (*20*). Furthermore, these data indicate that the spleen is not required as a source of circulating HSCs for BM cross engraftment.

Slightly increased chimerism of BM LT-HSCs was observed after longer intervals of parabiosis. Whereas mice joined for 16 weeks (experiment 2) exhibited LT-HSC chimerism about equivalent to that of animals joined for only 7 weeks, two animals from a pair joined for 39 weeks showed 8.1 and 8.8% BM LT-HSC chimerism (Table 1, experiment 3).

Numerous cytokines and mediators of inflammation can increase the number of circulating progenitors and HSCs in the mouse [for review, see (*21*)]. To control for the

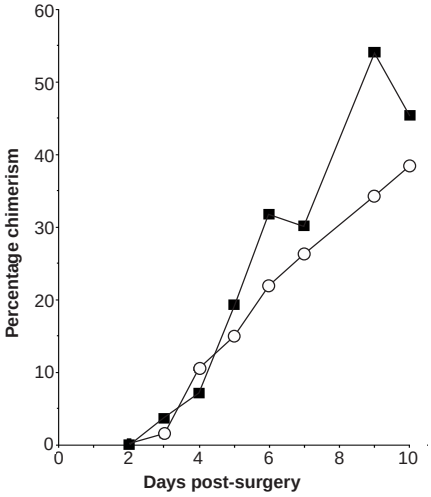

Fig. 1. Rapid establishment of cross circulation after parabiosis. Peripheral blood chimerism of parabiotic mice was determined by CD45 allotype analysis. Data were obtained by flow cytometry and are plotted as the percentage of partner-derived blood leukocytes for a representative pair. ■, CD45.1 cells in CD45.2 partner; ○, CD45.2 cells in CD45.1 partner.

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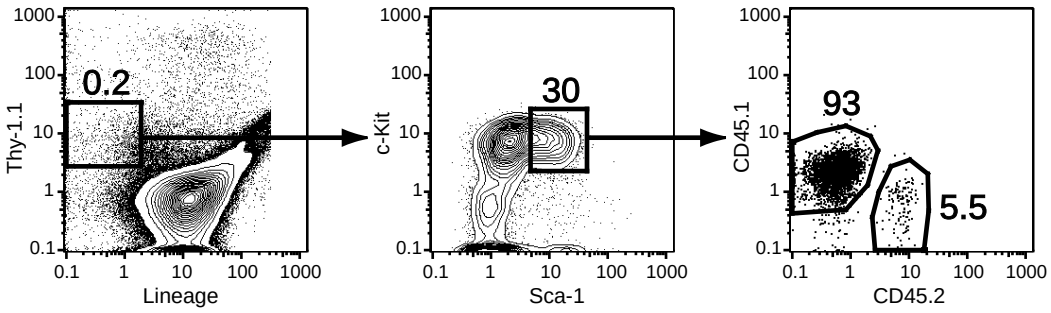

Fig. 2. Cross engraftment of partner-derived LT-HSCs in BM of parabiotic mice. Mice were joined for 7 weeks and separated 10 weeks before analysis. Contour and dot plots show staining profiles and gating of BM cells for CD45.1 and CD45.2 LT-HSC. Boxes and irregular polygons indicate gates—the left-hand plot shows whole BM, the center plot shows only Thy-1.1^{lo}Lineage^{-} cells, and the right-hand plot shows only Thy-1.1^{lo}Lineage^{-}Sca-1^{+}c-Kit^{+} cells (LT-HSCs). Numbers within plots represent the percentages of cells in the plot falling in gated areas.

possibility that enhanced mobilization of LT-HSCs could be induced by parabiosis surgery or by the state of parabiosis, we compared the LT-HSC content of blood obtained from parabiotic pairs with that of blood from unmanipulated animals (*22*, *23*) (Web fig. 1) (*24*). No significant differences were observed in the levels of donor cell engraftment of animals competitively repopulated with parabiont blood (collected 24 hours, 72 hours, or 10 days after surgery) or with control blood. These data strongly suggest that enhanced LT-HSC mobilization is not required for BM cross engraftment, although we cannot exclude a potential effect of parabiosis on the kinetics of HSC exchange between marrow and blood.

To estimate the magnitude of hematopoietic stem and progenitor cell flux through the blood, we determined the rate of disappearance from circulation of intravenously transferred day 12 colony-forming units–spleen (CFU-S$_{12}$) (*25*). In the BM, CFU-S$_{12}$ colonies derive from LT-HSCs, ST-HSCs, multipotent progenitors (MPPs), common myeloid progenitors (CMPs), and megakaryocyte-erythroid progenitors (MEPs) (*9*, *26*–*28*). In blood, the majority of CFU-S$_{12}$ activity is contained within the Sca-1^{-} fraction of blood cells, which is relatively enriched for myeloid committed progenitors (CMPs and MEPs) and depleted of Sca-1^{+} HSCs; however, a low level of CFU-S$_{12}$ activity is also detected in the Sca-1^{+} fraction of blood (Web fig. 2) (*24*).

To assay the blood residence time of multipotent and oligopotent progenitors and HSCs, we obtained lineage-depleted (Lin^{-}) peripheral blood cells (*29*) from 10 primary donor mice and injected them intravenously into anesthetized primary recipients, whose blood was sampled through the tail vein before injection (prebleed) and at two time points (30 s and 6 min) after cell transfer. To measure CFU-S$_{12}$ clearance, we injected collected blood from the primary recipient into lethally irradiated secondary recipient mice and counted spleen colonies in secondary recipients 12 days later. 92.8% of CFU-S$_{12}$ activity contained in peripheral blood of donor mice was cleared from the circulation within 30 s, and 99.6% was cleared within 6 min (Fig. 3) (*30*) (see also Web fig. 3) (*24*).

Table 1. Stable engraftment of BM of unconditioned partners by LT-HSCs after 7 weeks of parabiosis. Pairs of mice were parabiosed for 7, 16, and 39 weeks in experiments 1, 2, and 3, respectively. In experiment 1, pairs were surgically separated for 7, 10, or 22 weeks before analysis. Data are presented as percentage of partner-derived granulocytes in peripheral blood (PB) or as percentage of partner-derived LT-HSC in BM. No statistically significant differences in granulocyte or LT-HSC chimerism of CD45.1 versus CD45.2 mice were observed.

	Pair no.	Weeks after separation	Tissue analyzed	Chimerism (%)	
				CD45.1 partner	CD45.2 partner
Experiment 1	1	7	PB	3.2	1.3
		22	PB	3.1	1.2
		22	LT-HSCs	4.1	1.0
	2	7	PB	*	11†
		22	PB	*	15†
		22	LT-HSCs	*	20†
	3	7	PB	5.2	0.3‡
	4	7	PB	2.9	0.9
		22	PB	2.5	4.0
		22	LT-HSCs	3.3	2.9
	5	7	PB	3.7	4.3
		10	LT-HSCs	5.5	0
Experiment 2	1	0	LT-HSCs	§	0.5
	2	0	LT-HSCs	3.2	0.3
	3	0	LT-HSCs	6.6	0
Experiment 3	1	0	LT-HSCs	8.8	8.1

*Animal died before the date of analysis. †The partner of this animal was splenectomized before parabiosis surgery. ‡This animal was splenectomized before parabiosis surgery. §Sample lost.

These data suggest rapid flux of HSCs/progenitor cells in the circulation of normal animals.

On the basis of phenotypic and functional analyses, we estimate that there are 100 to 400 LT-HSCs in the blood of a mouse (*17*) (Web fig. 1) (*24*). This pool of blood-borne HSCs is relatively stable in frequency (Web fig. 4) (*24*), indicating that the flux of HSCs into the blood from BM and other tissues and the flux of HSCs out of the blood must be essentially the same. Thus, the rapid clearance from circulation that we observed for injected stem/progenitor cells may imply that large numbers of HSC pass through the bloodstream each day. However, our data do not exclude the possibility that a substantial component of this clearance rate may relate to an exchange of cells between a freely circulating state and a "marginated" state, in which HSCs are effectively removed from circulation but remain in the vascular space through adherence to lumenal endothelial surfaces. Marginated HSCs could immediately extravasate into tissue or, alternatively, could release from the vessel wall to reenter the freely circulating pool. In this circumstance, each blood-borne HSC likely transits into and out of circulation several times, thereby reducing the absolute number of individual HSCs in the circulating pool. Thus, the rapid clearance of blood-borne HSCs from the free circulation does not necessarily imply eqivalent migration into and out of the marrow, and the marrow to blood influx rate of HSCs, as well as the fraction of blood-borne HSCs that are derived from marrow versus extramedullary tissues, must still be determined.

Although a complete description of the potential functions of blood-borne HSCs and oligolineage hematopoietic progenitors will require further investigation, it is clear from the above data that at least some of these cells are capable of reengrafting BM. This possibility has been suggested by Quesenberry and colleagues, who reported high levels of donor BM chimerism after intravenous injection of large numbers of BM cells into unconditioned hosts (*31*, *32*).

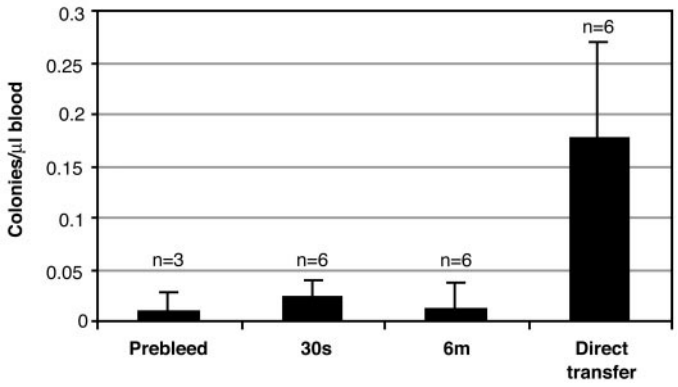

Fig. 3. Rapid clearance of peripheral blood CFU-S_{12} activity. Lineage-depleted peripheral blood cells collected from 10 primary donor mice were injected intravenously through the retroorbital sinus into anesthetized primary recipients, whose blood was sampled through the tail vein before injection ("prebleed") and at two time points after injection (30 s and 6 min). Blood collected from the primary recipient was injected intravenously into lethally irradiated secondary recipient mice, and spleen colonies in secondary recipients were counted 12 days later. As a control, 5% of the lineage-depleted peripheral blood cells from the primary donor were injected directly into irradiated recipients ("direct transfer"), bypassing the primary recipient, so that the starting CFU-S_{12} content of the primary donors' blood could be determined. Data are presented as the mean number (±SD) of colonies per μl of transferred blood. n = number of secondary recipients transplanted in each group. Differences between "direct transfer" and each of the other experimental groups were statistically significant ($P < 0.05$).

Our findings in parabionts considerably extend these observations, indicating that HSC engraftment of unconditioned BM does not depend on infusion of massive numbers of cells into the bloodstream and that cell turnover among BM HSC niches in normal animals is likely greater than previously appreciated. HSC migration may be important for redistribution of LT-HSCs within the BM compartment and may help to maintain hematopoietic homeostasis by insuring that HSC BM niches are not left unoccupied after HSC death or differentiation. Migratory HSCs may also function as an immediately available pool that can be rapidly recruited for extramedullary hematopoiesis after catastrophic blood loss.

The recent demonstration that small numbers of highly enriched BM-derived LT-HSCs give rise to hepatocytes in a liver injury model (*33*) suggests an additional potential function of blood-borne HSCs as a source of pluripotent or multipotent stem cells available for the repair of nonhematopoietic, as well as hematopoietic, tissues. Likewise, the constitutive transit of functional HSCs in the circulation of normal animals suggests caution in the interpretation of recent data regarding the generation of blood cells from nonhematopoietic tissues (*34*–*36*), as these tissues are likely to be contaminated with circulating HSCs. Even at low frequency, highly potent, itinerent HSCs could contribute substantially to hematopoietic readouts in assays designed to evaluate the "transdifferentiation" potential of nonhematopoietic tissues.

Finally, the existence of a physiological process by which HSCs and progenitors travel from BM to blood and back to functional niches in BM provides a rationale for the success of BM transplantation; the injected cells likely home to BM niches in irradiated recipients because the mechanisms underlying this migration preexist in unmanipulated animals. These data also suggest a modification of current thinking regarding mechanisms of induced HSC and progenitor cell mobilization, as "mobilizing" agents may function not only by increasing HSC emigration from BM but also by inhibiting normal reentry of these cells into tissues from the blood. Given the potential importance of the constitutively circulating pool of HSCs, further investigation of blood-borne HSCs phenotype, function, and evolutionary significance is of substantial interest.

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6. For parabiosis, mice were anesthetized to full muscle relaxation with xylazine and ketamine HCl (1.67 mg per 10 g of body weight) or with 2.5% avertin (15 μl per g of body weight), by intraperitoneal injection and joined by a modification of the technique of Bunster and Meyer (*7*). After shaving the corresponding lateral aspects of each mouse, matching skin incisions were made from the olecranon to the knee joint of each mouse, and the subcutaneous fascia was bluntly dissected to create about 1/2 cm of free skin. The olecranon and knee joints were attached by a single 2-0 silk suture and tie, and the dorsal and ventral skins were approximated by staples or continuous suture. In some experiments, after an interval of several weeks, parabiosed mice were surgically separated by a reversal of the above procedure. All protocols were approved by the Administrative Panel on Laboratory Animal Care (A-PLAC) at Stanford University School of Medicine.
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12. Monoclonal antibodies (mAb) used in immunofluorescence staining were prepared from hybridomas and included 19XE5 (mouse antibody to mouse Thy-1.1), 2B8 (rat antibody to mouse c-Kit), E13 (rat antibody to mouse-Sca-1, Ly6A/E), AL1-4A2 (mouse antibody to mouse CD45.2), and A20.1.7 (mouse antibody to mouse CD45.1). Lineage marker mAb included KT31.1 (rat antibody to mouse CD3), GK1.5 (rat antibody to mouse CD4), 53-7.3 (rat antibody to mouse CD5), 53-6.7 (rat antibody to mouse CD8), Ter119 (rat antibody to mouse erythrocyte specific antigen), 6B2 (rat antibody to mouse B220), 8C5 (rat antibody to mouse Gr-1), and M1/70 (rat antibody to mouse Mac-1). Fc receptors were blocked with 2.4G2 (rat antibody to mouse FcR) and with normal rat serum, as appropriate.
13. Cells were analyzed by multiparameter flow cytometry on either a modified two-laser FACS Vantage (Becton Dickinson Immunocytometry Systems, Mountain View, CA) or a modified three-laser cytometer (Cytomation, Fort Collins, CO, and Becton Dickinson), made available through the Flow Cytometry Shared User Group at Stanford University. Flow cytometry data were analyzed with FloJo software (Treestar, San Carlos, CA).
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19. Splenectomies were performed at least 3 weeks before parabiosis. Anesthetized mice were shaved on the left side and skin, and peritoneal incisions were made over the spleen with standard aseptic technique. Splenic vessels were tied off with 4-0 silk suture, and the spleen was carefully excised. The peritoneum was closed with 4-0 silk suture, and the skin closed with surgical skin staples. Protocols were approved by A-PLAC.
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29. Cells were stained on ice in Hank's balanced salt solution/2% calf serum (SM, staining medium) with phycoerythrin (PE) conjugates of the lineage marker antibodies listed above at previously optimized antibody concentrations for 25 min, rinsed twice in SM, and stained at 4°C for 20 min with SM-washed sheep antibody to PE-conjugated magnetic beads (Miltenyi Biotec, Auburn, CA) at 40 μl of beads per 10^8 cells. Lineage$^+$ cells were then depleted with the AutoMacs magnetic bead automated cell separation system (Miltenyi).
30. Total spleen colonies in secondary recipients were counted and normalized to the volume of blood transferred from the primary recipient (colonies per μl). To obtain the initial CFU-S_{12} frequency in the lineage-depleted donor cell population, we transferred 5% of the lineage-depleted cells directly to secondary recipients and normalized colonies counted on day 12 for the representative volume of blood that would have been transferred from the primary recipient to yield 5% of donor CFU-S_{12}

if no clearance occurred (i.e., 5% of estimated blood volume of the primary recipient animal, usually ~2.5 ml based on weight, divided by the number of "direct transfer" transplant recipients). Data were analyzed by Kruskal-Wallis one-way nonparametric analysis of variance and Mann-Whitney U tests, and differences between the "direct transfer" group and the "prebleed," "30 s," and "6 min" groups were statistically significant ($P < 0.05$).

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37. The authors thank L. Jerabek for superb laboratory management and for parabiosis surgery and training, V. Braunstein and S. Smith for antibody production, and L. Hidalgo and B. Lavarro (deceased) for animal care. We also thank M. Kondo for careful reading of the manuscript and helpful suggestions and M. Feldman for statistical advice. This work was supported by NIH grant 5R01 HL-58770 to I.L.W. D.E.W. was supported by National Institute of Allergy and Infectious Diseases Training Grant 5T32 AI-07290. A.J.W. was supported by American Cancer Society grant PF-00-017-01-LBC.

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T Cell Responses Modulated Through Interaction Between CD8αα and the Nonclassical MHC Class I Molecule, TL

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The thymus leukemia antigen (TL) is a nonclassical class I molecule, expressed abundantly on intestinal epithelial cells. We show that, in contrast to other major histocompatibility complex (MHC) class I molecules that bind CD8αβ, TL preferentially binds the homotypic form of CD8α (CD8αα). Thus, TL tetramers react specifically to CD8αα-expressing cells, including most intestinal intraepithelial lymphocytes. Compared with CD8αβ, which recognizes the same MHC as the T cell receptor (TCR) and thus acts as a TCR coreceptor, high-affinity binding of CD8αα to TL modifies responses mediated by TCR recognition of antigen presented by distinct MHC molecules. These findings define a novel mechanism of lymphocyte regulation through CD8αα and MHC class I.

Several nonclassical class I molecules are encoded in the *T* region of the mouse MHC. These proteins are antigens and are named after the thymus leukemia antigen (TL) encoded by the *T3/T18* gene pair (*1*). It is striking that TL displays nearly exclusive expression on epithelial cells of the small intestine (*2*). The expression by intestinal epithelial cells has led to the hypothesis that TL could be recognized by TCRs expressed on intraepithelial lymphocytes (IELs) (*3*). IELs are an enigmatic subset of predominantly $CD8^+$ T lymphocytes, which reside among epithelial cells. The unique location of these cells suggests that they may function in host defense, surveillance for damaged epithelium, or immune regulation.

To identify T cells that might interact with TL, we generated TL tetramers using a baculovirus expression system (*4*). As shown in Fig. 1A, TL tetramers stained the majority of IELs, but not splenocytes, and only a small minority of thymocytes. Tetramer binding was independent of TCR specificity, and it bound TCRαβ and TCRγδ cells equally well (*5*). Thus, expression of the TL receptor by IELs was distinct from the TCR. The staining of IELs with insect cell–derived TL indicates that tetramer binding was also independent of peptide loading to TL, consistent with previous evidence that TL does not bind peptides (*6*).

The α3 domain of TL conserves the CD8α-binding motif defined for class I molecules (*7*). In light of the specific binding to IELs, which express the homodimeric form of CD8, CD8αα, we reasoned that TL tetramers might bind this invariant molecule. Consistent with this, IELs from $CD8\alpha^{-/-}$ mice showed an almost complete absence of staining with the TL tetramer, whereas no reduction was observed on IELs from $CD8\beta^{-/-}$ mice (Fig. 1A). Similarly, thymocytes from $CD8\beta^{-/-}$ mice, which in the absence of CD8β express CD8αα homodimers, showed elevated TL tetramer binding, as did the few remaining $CD8^+$ splenocytes (Fig. 1A). Collectively, these data suggest that CD8αα, but not CD8αβ, forms a specific receptor for the TL tetramer.

The $CD8^-CD3^-$, TCR-deficient BW5147 thymoma did not stain with TL tetramers unless first transfected with CD8α (Fig. 1B), providing further evidence that tetramer binding is not TCR-dependent or IEL-specific. Similarly, transfectants of the T cell hybridoma BI-141 expressing CD8α alone reacted with the TL tetramer, whereas cells expressing CD4 did not (Fig. 1C). An antibody against TL (Fig. 1B), as well as an antibody against CD8α (Fig. 1C), could inhibit tetramer binding. Unlike $CD8\alpha\beta^+$ splenocytes, the TL tetramer bound the $CD8\alpha\beta^+$ transfectants too (Fig. 1C). However, multistep reciprocal immunoprecipitations (*8*) revealed that large numbers of CD8αα molecules were coexpressed with CD8αβ (Fig. 1D) and suggest that CD8αα might also be coexpressed on the TL tetramer binding $CD8\alpha\beta^+$ IELs (*5*).

To confirm the specific interaction of TL with CD8αα, direct binding studies were performed by surface plasmon resonance (*9*). TL monomer binding to CD8αα immobilized on a biosensor chip (*10*) exhibited fast association and disassociation rates, with an equilibrium-binding constant (K_D) of 12 μM (Fig. 2A). By contrast, saturation of TL binding with CD8αβ could not be reached at the highest concentration of TL (Fig. 2B). Consequently, an accurate K_D value could not be determined, although Scatchard analysis indicated a value of at least 90 μM (*11*). The class I molecule K^b did not show such a propensity and, in agreement with previous results, (*10*) bound with comparable affinity to CD8αα and CD8αβ (Fig. 2). These data directly demonstrated a preferential and relatively high affinity binding of TL to CD8αα.

We used a CD8α-deficient T cell hybridoma specific for SIINFEKL/H-$2K^b$ (*12*), and a CD8α-transfected variant, to examine the effects of CD8αα-TL binding on TCR-mediated responses (*13*). OVA peptide-loaded RMA-H (TL^-) thymoma cells, or TL-transfected variants, were used to stimulate the T cells. Upon antigen activation by CD8αα-expressing target cells, TL^+ stimulator cells showed a significant-

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