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Mast cell diversion of T-lineage precursor cells by the essential T-lineage transcription factor GATA-3

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Abstract

GATA-3 is essential for T cell development from the earliest stages. However, highly abundant GATA-3 can drive T-lineage precursors to a non-T fate, depending on Notch signaling and developmental stage. GATA-3 overexpression blocked pro-T cell survival when Notch-Delta signals were present, but enhanced viability in their absence. In double-negative (DN1) and DN2 but not DN3 fetal thymocytes, GATA-3 overexpression rapidly induced mast cell lineage respecification with high frequency by direct transcriptional reprogramming. Normal DN2 thymocytes also displayed mast cell potential, when interleukin 3 and stem cell factor were added in the absence of Notch signaling. Our results suggest a close relationship between the pro-T and mast cell programs and a new role for Notch in T-lineage fidelity.

INTRODUCTION

Within the hematopoietic system, the transcription factor GATA-3 is generally considered to be T-lineage specific and essential for T cell differentiation from the earliest stages[1–3]. GATA-3 is used recursively throughout T cell development (reviewed in [4]) and is a master regulator of T_H2 differentiation[5]. When Notch-Delta signaling triggers T-lineage differentiation from hematopoietic precursors, *Gata3* is among the earliest regulatory genes induced[6,7]. Thus GATA-3 is an attractive candidate for a role near the top of a T cell specification hierarchy.

Nevertheless, several problems have complicated attempts to detect the molecular mechanisms through which GATA-3 promotes T-lineage differentiation[8–12]. High doses of GATA factors block cell cycle progression, and GATA-3 itself can be antiproliferative in various T-lineage cells unless a hypomorphic mutant is used[13–16] (T.T. *et al*., unpublished data). However, a more radical possibility is that GATA-3 does not specifically instruct cells to adopt T-lineage identity, but only supports T cell development when expressed within a specific regulatory context. Here, we have revisited the question by asking more broadly what instructive impacts GATA-3 may have on developing prethymic and intrathymic lymphoid precursors when viability problems are circumvented. Both Notch-dependent and Notch-independent roles of GATA-3 have been assessed, using the continuous monitoring

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available in monolayer culture systems with OP9 stromal cells (OP9-control) or OP9 cells transfected with the Delta-class Notch ligand Delta-like 1 (OP9-DL1)[17].

Our results show that GATA-3 does not necessarily confer T-lineage identity, but can efficiently divert T-lineage precursors to an alternative, coherent developmental program. The early T-lineage role of GATA-3 is thus shown to be profoundly context- and dose-dependent. Overexpressed GATA-3 was antagonistic, not synergistic, with Notch-Delta signaling for pro-T cell survival. Yet, elevated GATA-3 conferred the gain of a different function, rapidly and efficiently promoting mast cell differentiation. This mast cell lineage response is available to a high fraction of thymocytes during stages prior to the c-Kit^{lo}CD44^{lo}CD25^{+} (DN3) stage. In these stages the cells also retain some natural killer (NK), dendritic, and macrophage developmental potential[18–22], but we show that the diverted thymocytes took a direct route toward mast cell differentiation quite distinct from any of these developmental pathways. Furthermore, we show that pro-T cells with unmanipulated GATA-3 expression also could access the mast cell differentiation pathway, albeit at a lower frequency. The results illuminate a new aspect of the T-lineage commitment process and reveal an unexpectedly intimate link between the T cell and mast cell developmental programs.

Results

GATA-3 blocks initiation of T-cell development

Since GATA-3 is one of the earliest T cell specific genes induced by Notch-DL1 stimulation of fetal liver (FL) hematopoietic precursor cells[6,7], we initially tested whether GATA-3 is the principal effector of T-lineage differentiation activated by Notch, or, whether GATA-3 promotes T cell development only in synergy with continuing Notch-Delta signals. In either case, the kinetics of GATA-3 induction could be rate-limiting for initiation of T cell development, and exogenous GATA-3 might either bypass the requirement for Notch signaling or accelerate the response to it. Despite possible antiproliferative effects, any T-lineage inducing activity should be detectable by transducing GATA-3 into purified hematopoietic precursors, and using the OP9 culture system to track the early time course of their differentiation in the presence or absence of DL1.

T-lineage promoting activity of GATA-3 might be seen in stem cells, accelerating them toward a lymphoid-restricted progenitor state, or in cells that are already lymphoid-restricted. Therefore, we tested both purified lymphoid-enriched progenitors (Kit^{+}Sca-1^{-}Lin^{-}CD27^{+}CD135^{+}CD127^{+}, ‘LEP’ hereafter) and more primitive hematopoietic precursors (Lin^{-}Sca-1^{+}Kit^{+}, LSK) from FL (Fig. 1a). Both populations generated CD19^{+} B-lineage cells in OP9-control culture and T-lineage cells on OP9-DL1, progressing from the CD44^{+}CD25^{-} DN1 stage to the CD44^{+}CD25^{+} DN2 and CD44^{-}CD25^{+} DN3 stages within about 7 days (Fig. 1b). As expected, the LEPs reached these lymphoid benchmarks more rapidly than LSKs (Fig. 1b, compare day 2, day 7).

However, when GATA-3 was introduced using a bicistronic retroviral vector, we found that neither prethymic precursor population showed enhanced early T-lineage differentiation after transduction with GATA-3 as compared to empty vector controls (LZRS), using vector-encoded EGFP expression to distinguish retrovirally transduced from nontransduced cells. GATA-3 transduction reduced cell yield in both OP9-control and OP9-DL1 cultures (Fig. 1c, CD45^{+}EGFP^{+} relative to EGFP^{-} cells) and blocked appearance of Thy-1^{+}CD25^{+} cells. Data shown are for Bcl2-transgenic precursors, which gave better cell recovery, but wild-type cells yielded similar results (Supplementary Fig. 1 online). In the absence of Notch-DL1, GATA-3 was not sufficient to promote entry into the T cell pathway (Fig. 1c, left), although the severe cell losses indicated it efficiently blocked B cell development

(T.T., A. Arias, unpublished data). Moreover, even with appropriate Notch stimulation (OP9-DL1), GATA-3 did not accelerate T-lineage differentiation; instead, it severely inhibited T-lineage differentiation from both progenitor subsets. The CD44 and CD25 profiles of surviving GATA-3–transduced cells showed retarded development and cell yields relative to LZRS-transduced cells. Therefore, in prethymic precursors, artificially elevated GATA-3 was neither sufficient nor permissive for T-lineage initiation even in collaboration with Notch signaling.

GATA-3 promotes thymocyte survival without Notch/DL1

Thymus entry and initiation of T cell differentiation do not protect cells from T-lineage inhibitory effects of GATA-3 overexpression since GATA-3 transduced fetal thymocytes were also inhibited in T-lineage differentiation on OP9-DL1 (Fig. 2a, left), confirming results from other assays[9,11]. Although most cells remained Thy-1$^+$, the yield of GATA-3 transductants and differentiation to the CD4$^+$CD8$^+$ double-positive (DP) stage (Fig. 2a, left bottom) were much reduced compared to LZRS-transduced control cells. GATA-3–transduced fetal thymocytes accumulated as DN1- and DN2-like cells with reduced CD25 expression (Fig. 2a, left). Similar developmental effects were seen using Bcl2-transgenic thymocytes, with less impact on viable cell yield (Supplementary Fig. 2 online).

However, intrathymic precursors displayed a positive response to GATA-3 overexpression. GATA-3 transduction enhanced proliferation and survival of thymocytes, provided that Notch–DL1 signaling was absent. This result contrasts with unmanipulated DN1–DN3 thymocytes which require continuous Notch-Delta interaction[7,20]. GATA-3 transduced thymocytes consistently expanded less than LZRS control transductants on OP9-DL1, but substantially more on OP9-control (Fig. 2a, right top; Fig. 2b). Furthermore, the cells increased in size (Fig. 2c, right), and included a subset with remarkably high c-Kit expression (Fig. 2c, right). These c-Kit^{hi} cells did not express myeloid markers and differed from normal c-Kit$^+$ thymocytes, lacking both Thy-1 and CD27 (Fig. 2c), a marker normally coexpressed with c-Kit on intrathymic progenitors[7,23–25].

c-Kit^{hi}CD27$^-$ cells were also seen among the survivors of GATA-3 transduced FL cells (not shown), but the survival advantage of GATA-3 transduced thymocytes in the absence of DL1 contrasted with the poor survival of GATA-3–transduced FL precursors under the same conditions. T-lineage priming or B-lineage inhibition by Notch-Delta signaling could underlie the difference (Fig. 2d), since a 5-day pre-culture of c-Kit$^+$CD27$^+$Lin$^-$ FL precursors in ‘T-lineage conditions’ (OP9-DL1), prior to LZRS or GATA-3 transduction, generated a population that responded to GATA-3 similarly to freshly isolated fetal thymocytes (compare Fig. 2a, Fig. 2d bottom). If returned to T-lineage conditions (OP9-DL1) after transduction, GATA-3 overexpression again severely reduced the frequency of EGFP$^+$ cells and blocked T cell development. But, the frequency of GATA-3 transduced cells increased and c-Kit$^+$CD27$^-$ cells were efficiently generated if the cells were cultured in the absence of Notch signaling after this priming step (Fig. 2d).

The ability of GATA-3 to inhibit growth in the presence of DL1 and to promote growth of c-Kit^{hi}CD27$^-$ cells in the absence of DL1 was similar to the effects of the known, essential mast cell factor GATA-2 (refs. 26–28), ectopically expressed in thymocytes (Supplementary Fig. 2). However, c-Kit^{hi} cell emergence did not depend on T-lineage inhibition directly, for the two effects could be separated. The hypomorphic KRR mutant of GATA-3 (refs. 15,16,29) was much less inhibitory of T cell development on OP9-DL1 than wild-type GATA-3 or GATA-2 (Supplementary Fig. 2; data not shown). However, it was equally effective at generating c-Kit^{hi}CD27$^-$Thy-1$^-$ cells from thymocytes in the absence of DL1 (Supplementary Fig. 2). Furthermore, the KRR mutant induced this developmental change

through gene expression effects closely matching those of wild-type GATA-3, described below (Supplementary Fig. 3 online).

GATA-3 redirects thymocytes to the mast cell lineage

The unusual c-Kit^{hi} population generated by GATA-3-transduced thymocytes was characterized further. To confirm whether c-Kit^{hi} cells can be derived from cells that have already initiated T-cell differentiation (Thy-1^{+}), C57BL/6 fetal thymocytes were transduced overnight with LZRS (Fig. 3a) or GATA-3 (Fig. 3b), and Thy-1^{+}GFP^{+} and Thy-1^{-}GFP^{+} cells were then sorted and placed in OP9-control culture. After 5 days, the cultured samples were resorted to separate different stages of response to GATA-3, based upon Thy-1 and c-Kit expression (Fig. 3a,b, right). GATA-3 transduction caused c-Kit^{hi} cells to emerge from both Thy-1^{+} (populations D, E) and Thy-1^{-} precursors (population G). To test whether these c-Kit^{hi} cells correspond to stem or multipotent progenitor cells, mast cells, or other hematopoietic cells, sorted populations denoted by A, B, C, etc. were tested in colony-forming assays (Table 1), mast cell culture conditions (Table 1, Fig. 3c–e), and gene expression analysis (below).

GATA-3-transduced thymocytes acquired *de novo* the abilities to form colonies in methylcellulose (Table 1) and to proliferate extensively in mast cell culture conditions containing interleukin 3 (IL-3) and stem cell factor (SCF; Table 1). These new growth properties were acquired whether cells were originally Thy-1^{-} or Thy-1^{+} (DN2 stage or later) at transduction, but were confined to those cells that had upregulated c-Kit by day 5 in response to GATA-3 (populations D, E and G in Table 1). LZRS-control transduced thymocytes after 5 day OP9-control coculture (populations A, B in Table 1) could not form colonies or grow detectably in mast cell cultures, and a distinct GATA-3–transduced Thy-1^{+}c-Kit^{-} subset (population C in Table 1) also could not grow in these conditions. The c-Kit^{+} GATA-3-transduced cells grown for approximately 2 weeks in mast cell culture conditions morphologically and phenotypically resembled large and granular c-Kit^{+}FcεRIα^{+} bone marrow-cultured mast cells (BMMCs) (Fig. 3c–e), distinct from normal thymocytes (Fig. 3f).

Pathway for thymocyte respecification by GATA-3

Mast cell lineage respecification by GATA-3 began immediately after transduction with GATA-3 (Fig. 4a,b). c-Kit upregulation began within one day after transduction, with and without Notch-DL1 signaling (Fig. 4a), whereas most empty-vector controls lost c-Kit while retaining CD27 expression, consistent with T-lineage identity. Distinct c-Kit^{hi}CD27^{lo}Thy-1^{lo} cells emerged from GATA-3-transduced cultures by day 3 with or without Notch-DL1 signaling, but more efficiently in its absence (Fig. 4a, red arrows). Further expansion of this c-Kit^{hi}CD27^{lo} population depended on DL1 absence.

In addition to c-Kit, GATA-3 upregulated expression of a full battery of mast cell specification genes within the first day. Such expression occurred without cell death in Bcl2-transgenic thymocytes and whether or not Notch signals were present (Fig. 4b). After transduction and 1 day OP9-control or OP9-DL1 co-culture, GATA-3 transduction caused ~30x elevation of *Gata3* RNA, and resulted in rapid upregulation of *Gata1* and *Gata2* (Fig. 4b, blue vs. pink bars). Notably, however, endogenous *Gata3* was not upregulated by GATA-3 (see Discussion). In addition to *Gata2* and *Gata1*, two other transcription factor genes required by mast cells, *Mitf* and *Tal1* (encoding transcription factor SCL), were also upregulated by GATA-3, as was the mast cell effector gene, carboxypeptidase (*Cpa3*). At the same time, pro-T cell transcription factor genes (*Tcf7*, encoding transcription factor TCF-1, and *Lef1*) and effector genes (*Il7ra*, *Rag1*) were downregulated. These changes did not reflect dedifferentiation or reversion toward a common myeloid precursor state, because

Sfpi1 (PU.1), a key determinant of myeloid potential in DN1 and DN2 pro-T cells, was initially downregulated by GATA-3 rather than upregulated (Fig. 4b). These early effects on T cell gene expression were remarkably selective. Numerous pro-T cell genes were relatively unaffected by GATA-3 overexpression at this time point, in the presence or absence of DL1 (Fig. 4b and Supplementary Fig. 3). These include *Cd3e* and *Cd3g*, *Lat* and genes encoding the transcription factors Myb, Ets, Ikaros, Gfi1, and bHLH E-proteins, all of which are rapidly downregulated when thymocytes are diverted to the myeloid or dendritic cell pathways[30].

Notch-DL1 signals moderated GATA-3 effects on many of its targets (Fig. 4b, dark blue vs. light blue bars). Several pro-T cell genes require continuous Notch signals to maintain their expression, but in most cases there was no evidence for interaction between GATA-3 and the Notch effects (Supplementary Figs. 3, 4 online). *Notch1*, *Notch3*, and Notch targets *Deltex1* and *Nrarp*, as well as pro-T genes, *Ptcra* (encoding pre-TCRα) in particular, required continuing Notch-Delta signaling for expression, with few or no GATA-3 effects in either condition (Fig. 4b and Supplementary Figs. 3, 4). However, the Notch target gene, *Hes1*, was upregulated by GATA-3 and fully protected by GATA-3 from downregulation if Notch signals were withdrawn (Fig. 4b, dark red vs. dark blue bars; Supplementary Fig. 3).

The upregulation of mast cell associated genes by GATA-3 appeared to be independent of mast cell cytokine signaling, as it occurred rapidly in the absence of exogenous IL-3 and was unaffected by addition of SCF (Supplementary Fig. 4). The immediate onset of these effects implies that GATA-3 itself was responsible, rather than the GATA-2 it can induce, since absolute amounts of GATA-2 at this time point remained relatively low (Supplementary Fig. 3). Thus, it appears that GATA-3 overexpression can directly and rapidly impose a new regulatory program in developing thymocytes.

Establishment of mast cell gene expression program

The initial gene expression changes after 1 day in culture progressively intensified over the next four days in OP9-control culture, specifically in cells that went on to express high amounts of c-Kit. The gene expression of cell populations obtained from 5 day OP9-control cultures was measured by quantitative RT-PCR (Fig. 4c), revealing specific differences between those GATA-3-transduced cells that were able to divert to the mast cell lineage (D and E, in gray boxes) and those which were unable to divert despite GATA-3 transduction (C), or LZRS-transduced controls (A and B; see Fig. 3 for populations). Day 5 effects were also compared with gene expression changes in GATA-3 transduced cells after a further 2 weeks of culture, either in Methocult (‘Colonies’) or in mast cell cytokines IL-3 and SCF (‘MC culture’), in which the cells fully convert to mast cell phenotype (cf. Fig. 3c, e, f). Because of the large dynamic range of effects, the results are displayed as the absolute gene expression obtained in these samples measured relative to β-actin, with gene expression in normal E14.5 fetal liver, fetal thymocytes, and bone marrow-derived mast cells (BMMC) shown for reference (Fig. 4c).

By day 5, mast cell transcription factor genes *Gata1*, *Gata2*, *Mitf* and *Tal1*, and effector gene *Cpa3*, were all upregulated in the c-Kit^{hi}Thy-1^{-} subset (Fig. 4c, light blue) to expression approaching or matching that of BMMC (Fig. 4c, black). The expression of another mast cell-specific effector gene encoding tryptase, *Mcpt6*, which was not upregulated at day 1 (Supplementary Fig. 3), also increased tremendously by this time. Mast cell specification depends on a collaborative interaction between GATA factors and moderate amounts of PU.1 (ref. 27), and despite the initial drop in PU.1 expression, c-Kit^{+} GATA-3 transductants stabilized PU.1 expression similar to or approaching that of BMMCs at this time point (Fig. 4c). c-Kit^{+} GATA-3 transductants also matched mast cells, rather than myeloid or erythro-megakaryocytic cells, in their low expression of C/EBPα (*Cebpa*),

hemoglobin β (*Hbb-b1*), Tpo-receptor (*Mpl*), and Epo-receptor (*Epor*), and their expression of regulatory genes such as *Fog1*, *Fli1*, c-*Myb*, *Ets2*, *Runx3*, and *Gfi1* (Supplementary Fig. 5 online). Thus, these changes quantitatively established a mast cell-like program within 5 days in lymphoid culture conditions (Fig. 4c, light blue). Little further increase in mast cell gene expression occurred in GATA-3-transductants grown in long-term IL-3–SCF cultures (Fig. 4c, purple).

GATA-3 transductants with mast cell lineage potential (D, E and long-term cultures) now lost expression of diverse pro-T cell genes in a reciprocal pattern to mast cell gene activation, not only *Tcf7* and *Lef1*, but also *Lck*, *Zap70* and others, approaching the phenotype of BMMCs. Furthermore, many Notch-dependent genes, including *Ptcra*, *Rag1*, *Deltex1*, and *Notch1* itself, were downregulated by culture on OP9-control due to loss of Notch signals (Fig. 4c, Supplementary Fig. 5 online). However, GATA-3 transductants maintained moderate expression of *Notch1* as well as of the Notch-responsive gene *Hes1*, even after 5-day absence of Notch–DL1 signaling, at amounts similar to those found in BMMCs. Interestingly, among pro-T cell genes, only *Cd3e* expression was maintained in all GATA-3 transductants derived from Thy-1$^+$ cells (Fig. 4c), contrasting with its complete absence in BMMCs. This remarkably stable *Cd3e* expression distinguished thymocyte-derived mast cells from normal BMMCs, and confirmed their T-lineage origin.

Although all GATA-3 transduced cells contained similar amounts of GATA-3 mRNA, ~10x higher than normal thymocytes (Fig. 4c, 'GATA-3 All'), its impact on other genes correlated with the ability or inability of each population to generate mast cells (cf. Table 1). c-Kit^{lo}Thy-1$^+$ cells (Fig. 4c, population C, dark blue) which cannot become mast cells in long-term culture, maintained gene expression profiles most like fetal thymocytes (light green), while gene expression patterns of c-Kit$^+$Thy-1$^-$ cells (E, light blue) most closely resembled that of BMMCs (black), with intermediate gene expression profiles in c-Kit$^+$Thy-1$^+$ cells (D, medium blue). These results suggest variations in cell responses to overexpression of GATA-3 (see below).

Thus, the subpopulations of GATA-3-transduced Thy-1$^+$ fetal thymocytes that can generate mast cells have already switched within 5 days to a gene expression profile comprehensively matching BMMCs rather than pro-T cells. Positive upregulation of mast cell transcription factor genes is among the earliest responses to GATA-3, providing regulatory partners that can cooperate to enforce the lineage switch. These changes in programming are driven by overexpression of GATA-3 before any selection in IL-3 and SCF, as summarized in Supplementary Fig. 6 online.

Developmental window and frequency of lineage shift

To determine whether initial developmental stage differences underlie these distinct responses to GATA-3, we purified DN1, DN2, and DN3 E14.5 C57BL/6 fetal thymocytes and compared their responses to GATA-3 or LZRS transduction (Fig. 5). GATA-3 transduction promoted development of Thy-1^{lo}c-Kit$^+$ cells (arrows, Fig. 5b, top) from both DN1 and DN2 starting populations. Both yields and c-Kit intensities were substantially higher in transductants cultured on OP9-control than on OP9-DL1. The phenotypes of EGFP$^+$ GATA-3 transduced cells sharply diverged from the EGFP$^-$ cells within the same samples, which remained overwhelmingly Thy-1$^+$ and progressed normally to c-Kit$^-$ cells (EGFP$^-$).

In contrast to DN1 and DN2 cells, DN3 cells failed to upregulate c-Kit after GATA-3 transduction and only responded by a partial Thy-1 downregulation on OP9-control stroma. Both c-Kit upregulation in DN1 and DN2 cells and Thy-1 downregulation in DN2 and DN3 cells were specific effects of GATA-3 transduction (compare Fig. 5a–d). When sorted

EGFP$^+$ cells from the OP9-control and OP9-DL1 cultures were cultured with IL-3 and SCF, mast cells developed and grew progressively from the DN1 and DN2 cells transduced with GATA-3, while DN3 cells had apparently lost the ability to undergo this fate conversion (Fig. 5a,b, numbers at right). Thus a developmental constraint, imposed between the DN2 and DN3 stages, sets the boundary for competence to transdifferentiate in response to GATA-3. This developmental boundary, the changes in gene expression that may contribute to it, and their relationship to Notch signaling effects are summarized in Supplementary Fig. 6 online.

The ability to be diverted by GATA-3 was common in the DN2 thymocyte population, as shown by precursor frequency analysis (Fig. 5e). Optimal conditions for observing mast cell conversion yielded precursor frequencies of 1/15-1/25 in GATA-3 transduced C57BL/6 DN2 thymocytes (blue curves). However, since lineage conversion entails a poorly characterized switch in survival requirements, we also tested Bcl2-tg DN2 cells, which showed substantially increased precursor frequencies of 1/2-1/3 (orange curves) when transduced with GATA-3. Thus, when interim survival is not an issue, GATA-3 is highly efficient in redirecting T-cell precursors into the mast cell lineage. Furthermore, untransduced Bcl2-tg DN2 cells showed low, but detectable, mast cell development (green curves).

Mast cell potential of unmanipulated thymocytes

The unexpected diversion of T cell progenitors into mast cells by GATA-3, and the observed low frequency mast-cell development from Bcl2-tg DN2 cells, urged us to investigate mast cell lineage potential of normal, unmanipulated thymocytes. Although known to be uncommitted, early T cell precursors have not been shown to have mast cell potential. We sorted the most immature fetal thymocytes, separating c-Kit$^+$Thy-1$^-$CD25$^-$ (pre-DN1 and DN1-like) and c-Kit$^+$Thy-1$^+$CD25$^+$ DN2 thymocytes (Fig. 6a); both demonstrated T-lineage potential with slower or faster kinetics, respectively, corresponding to their initial developmental stage (Fig. 6b). In the presence of Notch-DL1 signaling, no FcεRI$^+$c-Kit$^+$ cells developed, not even with exogenous SCF and IL-3. However, on OP9-control cells with exogenous SCF plus IL-3 as well as IL-7, mast cells emerged within 3–4 weeks. c-Kit$^+$Thy-1$^+$CD25$^+$ DN2 thymocytes could generate mast cells as shown by phenotype (Fig. 6c) and morphology (Fig. 6d,e), but surprisingly the more primitive c-Kit$^+$Thy-1$^-$CD25$^-$ thymocytes could not. Thus, even some normal fetal thymocytes possess mast cell-lineage potential, which is repressed by Notch1-DL1 signaling. Furthermore, this potential is most accessible in T cell precursors that have already initiated T-lineage specification, suggesting a close relationship between the initial developmental stages of both lineages.

DISCUSSION

GATA-3 is activated in response to Notch-Delta signaling as an early and essential event in T-lineage specification. We have shown here that GATA-3, if highly expressed, diverts thymocytes from the T lymphoid pathway by activating a mast cell-like program, independent of exogenous mast-cell growth factors IL-3 and SCF. Diversion occurs at the same stages of pro-T cell development when GATA-3 is normally required for the DN1 to DN2 transition, and is highly efficient, especially from DN2 thymocytes. Thus the positive roles of GATA-3 for T-lineage development are intrinsically dose-dependent. Abundant GATA-3 immediately activates mast cell regulatory and differentiation genes in pro-T cells, and handicaps viability in the presence of Notch-Delta signaling but actively promotes survival and proliferation when the Notch ligand DL1 is removed. Notch signals appear to moderate GATA-3 effects in part by blunting gene expression changes and sustaining expression of T cell genes, but also by blocking growth of cells overexpressing GATA-3. Consistent with this, some normal, unmanipulated DN2 thymocytes also displayed mast cell

lineage potential, but only in the absence of Notch-DL1 signaling and in the presence of mast cell supporting cytokines.

In vivo relationships between T lineage cells and mast cells have been suggested for a long time[31], but without evidence for a definite pathway. The striking ability of mast cells to express a broad range of 'T-lineage specific' cytokines, as well as GATA-3, implies some convergence of T and mast cell programming. The T cell marker Thy-1 identifies mast cell precursors in fetal blood, as Thy-1^{lo}c-Kit^{hi} cells displayed mast cell potential in contrast to their Thy-1^{-}c-Kit^{+} counterpart[32]. More recently, the thymus has been identified as a site of mast cell development in chicken embryos[33]. Mast cells have also been reported in human thymuses in pathological cases[34,35], and are seen in medullary regions which are poor in Delta-class Notch ligands[36,37]. Our results suggest that such mast cells could develop intrathymically, possibly amplified *in situ* by a breakdown of Notch-dependent restraints.

The major regulatory pathway for normal mast-cell differentiation is not well characterized. Balanced activity of PU.1 and GATA factors is known to be required, along with Mitf and possibly SCL, and roles of GATA-2 and GATA-1 in this process can now be distinguished[27,38–40]. However, it has remained obscure whether mast cells are 'myeloid' or in a distinct class of their own. They can be derived from precursors separate from most myeloid lineages[38,41]. Unlike monocyte and granulocyte lineages, but corresponding with lymphocytes, they develop through a pathway that excludes C/EBPα[42]. Thus, our results provide concrete evidence for one specific function – elevated GATA-2 or GATA-3 expression – that provides direct access to the mast cell pathway from an uncommitted, but differentiating, lymphoid precursor. We do not propose that the thymus is a major source of mast cells, but early developing T cells appear to be primed for mast cell development and may provide an excellent model system to dissect distinct regulatory stages of mast cell lineage commitment.

The mast cell lineage diversion of immature thymocytes is triggered by a factor that these cells express normally, namely GATA-3. High GATA-3 expression may affect target genes that can be regulated by GATA-2 in prethymic precursors, as both have similar effects. However, our results show that differences in absolute expression level are all that is needed to distinguish between GATA-3 contributions to the T cell lineage and GATA-3-driven mast cell lineage diversion. The ability of some DN2 cells, expressing barely detectable GATA-2, to differentiate into mast cells without exogenous GATA-3 suggests that their endogenous GATA-3 expression can extend into the mast cell supporting range.

The mast cell program only seems accessible to DN1 and DN2 stage thymocytes, which also preserve latent NK and dendritic cell or even macrophage developmental potentials. This finding raises the question whether lineage plasticity depends on initial retrogression to some common myeloid-lymphoid precursor state before adopting any of these alternative fates. If so, then the mast cell reprogramming would be an instance of this general lymphomyeloid plasticity[43]. In the case of the mast cell versus monocyte or dendritic cell alternatives, however, this possibility can be excluded. Diversion of pro-T cells to these different pathways can be triggered synchronously by elevating GATA-3 or PU.1, respectively, making it possible to compare the programs of regulatory change. The time courses of gene expression effects (compare ref.[30]) show that the two paths are completely distinct.

In the case of PU.1-mediated monocytic-dendritic respecification, most early effects are broadly to inhibit pro-T cell gene expression, including genes that encode signaling kinases and transcription factors Myb, HES-1, Gfi1, bHLH factors, and Ets factors as well as TCF-1; *Gata3* is not substantially affected[30]. PU.1 in this context upregulates its own

expression, along with weak upregulation of Id2 and a few myeloid genes that are much more strongly induced later[30]. In contrast, the dominant GATA-3 effect is the immediate upregulation of mast cell associated genes, including *Gata2*, long before most T-lineage genes are downregulated. GATA-3 upregulation has no effect whatsoever on *Myb*, *Gfi1*, or *Ets* factor genes, and acts as a positive regulator of *Hes1*, while transiently downregulating PU.1. The early upsurge of GATA-2, Mitf, and GATA-1 expression provides regulatory partners that may assist or replace GATA-3 for expression of later mast cell gene products, such as tryptase. Thus, GATA-3 immediately redirects immature thymocytes to a mast cell state without 'crossing paths' with the monocyte-dendritic programs controlled by PU.1 and/or C/EBPα[30,44]. In fact, the transient repression of PU.1 in GATA-3-transduced DN1 and DN2 cells may be important to block access to the monocyte or dendritic alternatives until the new regulatory state is established. Thus the mast-cell pathway, accessible to a few DN2 thymocytes naturally and to a large fraction of them when GATA-3 is elevated, represents a qualitatively distinct, additional option for pro-T cells.

Notch-Delta signaling restrains pro-T cell access to the mast-cell pathway. We show that Notch-Delta signaling enforces a survival penalty on pro-T cells overexpressing GATA-3, which is only partially counteracted by Bcl2 expression. At lower, endogenous expression abundance of GATA-3, in contrast, Notch and GATA-3 are jointly required for pro-T cell viability (D.D. Scripture-Adams and E.V.R., unpublished data). Notch-Delta signaling also blunts, but cannot prevent, the upregulation of mast cell genes by highly abundant GATA-3 expression. These antagonistic Notch effects on high GATA-3 activity are not mediated by HES-1 (cf. ref. [45]), because GATA-3 upregulates *Hes1* independently of Notch. HES-1 can promote precursor expansion[46], and it is possible that the selective upregulation of *Hes1* by GATA-3 contributes to its ability to enhance cell growth in the absence of Notch signaling. Other Notch-induced mediators must be responsible for penalizing GATA-3 overexpressing cells.

If a substantial increase in GATA-3 can reveal mast cell potential in DN1 and DN2 thymocytes, then the mechanisms normally restricting GATA-3 abundance *in vivo* must be robust to keep this potential obscured. However, each of the hematopoietic GATA genes has positive autoregulation potential, including GATA-3 itself in peripheral T cells[47–49], and presumably GATA-3 activates GATA-1 and GATA-2 in our studies by acting through their autoregulatory target sites. In pro-T cells, then, GATA-3 itself must be prevented from positive autoregulation, and our gene expression analysis supports this notion. The positive roles of GATA-3 in T-lineage differentiation may therefore depend paradoxically on damping functions that prevent autoactivation and thus maintain the uniquely balanced regulatory state of T-lineage precursors.

T-lineage commitment, at the DN3 stage, coincides with an increase in GATA-3 expression at the RNA level[22], as GATA-3 is required for β-selection[50], while the cells lose sensitivity to the mast cell diversion activity of GATA-3. The 'window' for susceptibility to diversion is likely to depend on specific gene regulatory features of DN1 and DN2 fetal thymocytes such as the persistence of PU.1 or SCL. In any case, our results suggest that diversion depends on the ability of GATA-3 to rapidly upregulate *Gata2*, *Scl/Tal-1* and *Mitf*. The T-lineage commitment process may therefore result from increasing restrictions on the accessibility of genes like these to activation, or reactivation, during a stage when high GATA-3 activity becomes vital for a new, TCR-dependent role.

METHODS

Monoclonal antibodies and flow cytometry

Monoclonal antibodies have been described previously[7,23], except for: CD19 (clone 6D5, PE and PE-Cy5), CD127 (clone A7R34, PE-Cy5), CD135 (A2F10, PE), FcεRIα (clone MAR-1, PE) and Sca-1 (clone D7, FITC), all from eBioscience. For detection of biotinylated antibodies, streptavidin-AlexaFluor405 (Invitrogen) was used. Stainings were performed after blocking Fc receptors as described[7,23]. Cell sorting was done on a FACSVantage SE or FACSAria (Becton Dickinson Immunocytometry Systems [BDIS]) and analysis on a FACSCalibur (BDIS). Data was acquired and analyzed using CellQuest Pro or Diva (BDIS) or FlowJo (TreeStar Inc.) software. All fluorescence is plotted on a four-decade logarithmic scale with forward scatter and cell numbers on a linear scale.

Fetal tissues

Embryonic day 14–14.5 C57BL/6, (C57BL/6xDBA/2)f1 (BDF1) and Eμ-Bcl2–25 (Bcl2-tg) mouse embryos were generated from our colony[30] or obtained from the Caltech Genetically Engineered Mouse facility. FL cells were depleted for lineage marker-positive cells as described[7,23]. Fetal thymus tissues were mechanically disrupted using a dounce homogenizer and the resulting cell suspension was filtered prior to staining. All animal protocols were reviewed and approved by the California Institute of Technology Institute Animal Care and Use Committee.

Retroviral gene transfer

GATA-3 and GATA-3-KRRm were inserted into the bicistronic LZRS retroviral vector containing an IRES-GFP and packaged using the Phoenix-Eco packaging cell line as described elsewhere[15]. Viral supernatant was collected and stored in aliquots at −70 °C until use. Transduction conditions were essentially as described[7,23]. Briefly, non-tissue culture plates (24 or 96-well, Corning) were coated with 24 μg/ml retronectin for 2 h at 20–25 °C, then blocked with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min and incubated with retroviral supernatant for 4 h at 37 °C. After coating, viral supernatant was removed and cells were added immediately in OP9 coculture medium as described[7,23], always containing 5 ng/ml of Flt3L and IL-7. Cells were then cultured on the virus-coated plates overnight (15–20 h) for transduction.

Cell culture conditions

Conditions for OP9-DL1 and OP9-control stromal cocultures have been described in detail[7,23]. All cytokines were from Peprotech and all tissue culture media were from Invitrogen except as indicated. Differentiation cultures contained 5 ng/ml each of Flt3L and IL-7, except for specific OP9 cocultures in which Flt3L was replaced by SCF (5 ng/ml), as indicated. In some experiments, substitution of SCF for Flt3L enhanced the detection of c-Kit$^+$ cells over a 5-day period, but this appeared to reflect enhanced survival as it had no detectable effect on the initial gene expression changes induced by GATA-3 (Fig. 4, Supplementary Fig. 4). Several different culture conditions were used for the generation of mast cells from pro-T cells. In experiments with sorted DN subsets (Fig. 5a–d), after OP9 culture GFP$^+$ sorted cells were cultured in 50 ng/ml IL-3 and 20 ng/ml SCF. For limiting dilution analysis (Fig. 5e) transduced or mock-transduced DN2 cells were sorted directly onto OP9-control cells with 5 ng/ml IL-7 plus 50 ng/ml IL-3 and 20 ng/ml SCF. Medium plus IL-3 and SCF, but not IL-7, was replaced after 1 week. For the generation of mast cells from normal thymocytes, cocultures contained 5 ng/ml of Il-7 and Flt3L or contained SCF (20 ng/ml) and IL-3 (50 ng/ml) with IL-7 and Flt3L. In this case, IL-7 and Flt3L were omitted from the cultures after 2 weeks. Methocult GF M3434 (StemCell Technologies),

containing SCF, IL-3, IL-6, and EPO, was used for colony-forming assays, according to the instructions of the manufacturer, with $10 – 25 \times 10^3$ cells of sorted transduced fetal thymocytes or $100 – 250 \times 10^3$ BM cells. Colonies were counted after 9 – 14 days of culture. Cells were harvested for RNA analysis by vigorously pipetting the semi-solid cultures with 10 ml of unsupplemented DMEM medium followed by centrifugation. For mast-cell-culture from Thy-1 and c-Kit sorted cells (Fig. 3), after 5 day coculture with OP9-control cells plus IL-7 and Flt3L, $10 – 100 \times 10^3$ sorted transduced fetal thymocytes or 10^5 BM cells were placed in DMEM supplemented with 10% fetal bovine serum, penicillin, streptomycin, L-glutamine, mixed 9:1 with WEHI-3B cell (ATCC) conditioned medium as a source for IL-3, plus 5 ng/ml SCF. Medium and cytokines were replaced after 7 days.

RNA extraction and quantitative real-time RT-PCR

Sorted cells were placed in 500 μL of Qiazol (Qiagen) and stored at −80 °C. RNA was extracted using RNeasy RNA isolation kits (Qiagen) and reverse transcribed into cDNA using Superscript RT II or III (Invitrogen). Real-time PCR reactions were performed using SYBRGreen Universal Master Mix on a GeneAmp 7700 sequence detection system (Applied Biosystems) with primers synthesized by Operon published previously[22,23,30] plus additional primers provided in Supplementary Table 1. Relative expression was calculated for each gene by the ΔCt method using β-actin for normalization. Standards were BMMC and unmanipulated E14.5 C57BL/6 fetal liver (FL) and fetal thymus.

Statistical methods

Geometric means and standard deviations were calculated using Microsoft Excel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Samson SI, et al. GATA-3 promotes maturation, IFN-γ production, and liver-specific homing of NK cells. Immunity. 2003; 19:701–711. [PubMed: 14614857]
2. Hendriks RW, et al. Expression of the transcription factor GATA-3 is required for the development of the earliest T cell progenitors and correlates with stages of cellular proliferation in the thymus. Eur J Immunol. 1999; 29:1912–1918. [PubMed: 10382753]
3. Ting CN, Olson MC, Barton KP, Leiden JM. Transcription factor GATA-3 is required for development of the T-cell lineage. Nature. 1996; 384:474–478. [PubMed: 8945476]
4. Rothenberg EV, Taghon T. Molecular Genetics of T Cell Development. Annu Rev Immunol. 2005; 23:601–649. [PubMed: 15771582]
5. Zhu J, Yamane H, Cote-Sierra J, Guo L, Paul WE. GATA-3 promotes Th2 responses through three different mechanisms: induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors. Cell Res. 2006; 16:3–10. [PubMed: 16467870]

6. Höflinger S, et al. Analysis of Notch1 function by *in vitro* T cell differentiation of *Pax5* mutant lymphoid progenitors. J Immunol. 2004; 173:3935–3944. [PubMed: 15356142]
7. Taghon TN, David ES, Zúñiga-Pflücker JC, Rothenberg EV. Delayed, asynchronous, and reversible T-lineage specification induced by Notch/Delta signaling. Genes Dev. 2005; 19:965–978. [PubMed: 15833919]
8. Chen D, Zhang G. Enforced expression of the GATA-3 transcription factor affects cell fate decisions in hematopoiesis. Exp Hematol. 2001; 29:971–980. [PubMed: 11495703]
9. Taghon T, et al. Enforced expression of GATA-3 severely reduces human thymic cellularity. J Immunol. 2001; 167:4468–4475. [PubMed: 11591773]
10. Nawijn MC, et al. Enforced expression of GATA-3 during T cell development inhibits maturation of CD8 single-positive cells and induces thymic lymphoma in transgenic mice. J Immunol. 2001; 167:715–723. [PubMed: 11441075]
11. Anderson MK, et al. Definition of regulatory network elements for T-cell development by perturbation analysis with PU.1 and GATA-3. Devel Biol. 2002; 246:103–121. [PubMed: 12027437]
12. Heavey B, Charalambous C, Cobaleda C, Busslinger M. Myeloid lineage switch of Pax5 mutant but not wild-type B cell progenitors by C/EBPα and GATA factors. EMBO J. 2003; 22:3887–3897. [PubMed: 12881423]
13. Zhu J, et al. Growth factor independent-1 induced by IL-4 regulates Th2 cell proliferation. Immunity. 2002; 16:733–744. [PubMed: 12049724]
14. Ezoe S, et al. GATA-2/estrogen receptor chimera regulates cytokine-dependent growth of hematopoietic cells through accumulation of p21(WAF1) and p27(Kip1) proteins. Blood. 2002; 100:3512–3520. [PubMed: 12393444]
15. Hernández-Hoyos G, Anderson MK, Wang C, Rothenberg EV, Alberola-Ila J. GATA-3 expression is controlled by TCR signals and regulates CD4/CD8 differentiation. Immunity. 2003; 19:83–94. [PubMed: 12871641]
16. Yamagata T, et al. Acetylation of GATA-3 affects T-cell survival and homing to secondary lymphoid organs. EMBO J. 2000; 19:4676–4687. [PubMed: 10970860]
17. Zúñiga-Pflücker JC. T-cell development made simple. Nat Rev Immunol. 2004; 4:67–72. [PubMed: 14704769]
18. Shortman K, et al. The linkage between T-cell and dendritic cell development in the mouse thymus. Immunol Rev. 1998; 165:39–46. [PubMed: 9850850]
19. Shen HQ, et al. T/NK bipotent progenitors in the thymus retain the potential to generate dendritic cells. J Immunol. 2003; 171:3401–3406. [PubMed: 14500634]
20. Schmitt TM, Ciofani M, Petrie HT, Zúñiga-Pflücker JC. Maintenance of T cell specification and differentiation requires recurrent Notch receptor-ligand interactions. J Exp Med. 2004; 200:469–479. [PubMed: 15314075]
21. Balciunaite G, Ceredig R, Rolink AG. The earliest subpopulation of mouse thymocytes contains potent T, significant macrophage, and natural killer cell but no B-lymphocyte potential. Blood. 2005; 105:1930–1936. [PubMed: 15522952]
22. David-Fung ES, et al. Progression of regulatory gene expression states in fetal and adult pro-T cell development. Immunol Rev. 2006; 209:212–236. [PubMed: 16448545]
23. Taghon T, Yui MA, Pant R, Diamond RA, Rothenberg EV. Developmental and molecular characterization of emerging β- and γδ-selected pre-T cells in the adult mouse thymus. Immunity. 2006; 24:53–64. [PubMed: 16413923]
24. Allman D, et al. Thymopoiesis independent of common lymphoid progenitors. Nat Immunol. 2003; 4:168–174. [PubMed: 12514733]
25. Igarashi H, Gregory SC, Yokota T, Sakaguchi N, Kincade PW. Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. Immunity. 2002; 17:117–130. [PubMed: 12196284]
26. Tsai FY, Orkin SH. Transcription factor GATA-2 is required for proliferation/survival of early hematopoietic cells and mast cell formation, but not for erythroid and myeloid terminal differentiation. Blood. 1997; 89:3636–3643. [PubMed: 9160668]

27. Walsh JC, et al. Cooperative and antagonistic interplay between PU.1 and GATA-2 in the specification of myeloid cell fates. Immunity. 2002; 17:665–676. [PubMed: 12433372]
28. Iwasaki H, et al. The order of expression of transcription factors directs hierarchical specification of hematopoietic lineages. Genes Dev. 2006; 20:3010–3021. [PubMed: 17079688]
29. Takemoto N, Arai K, Miyatake S. Cutting edge: the differential involvement of the N-finger of GATA-3 in chromatin remodeling and transactivation during Th2 development. J Immunol. 2002; 169:4103–4107. [PubMed: 12370337]
30. Franco CB, et al. Notch/Delta signaling constrains re-engineering of pro-T cells by PU.1. Proc Natl Acad Sci USA. 2006; 103:11993–11998. [PubMed: 16880393]
31. Burnet FM. The probable relationship of some or all mast cells to the T-cell system. Cell Immunol. 1977; 30:358–360. [PubMed: 67911]
32. Rodewald HR, Dessing M, Dvorak AM, Galli SJ. Identification of a committed precursor for the mast cell lineage. Science. 1996; 271:818–822. [PubMed: 8629001]
33. Crivellato E, Nico B, Battistig M, Beltrami CA, Ribatti D. The thymus is a site of mast cell development in chicken embryos. Anat Embryol (Berl). 2005; 209:243–249. [PubMed: 15712013]
34. Wise WS, Still WJ, Joshi VV. Severe combined immunodeficiency with thymic mast cell hyperplasia. Arch Pathol Lab Med. 1976; 100:283–286. [PubMed: 946756]
35. Murphy M, Friend DS, Pike-Nobile L, Epstein LB. Tumor necrosis factor-α and IFN-γ expression in human thymus. Localization and overexpression in Down syndrome (trisomy 21). J Immunol. 1992; 149:2506–2512. [PubMed: 1388194]
36. Visan I, Yuan JS, Tan JB, Cretegny K, Guidos CJ. Regulation of intrathymic T-cell development by Lunatic Fringe- Notch1 interactions. Immunol Rev. 2006; 209:76–94. [PubMed: 16448535]
37. Mohtashami M, Zúñiga-Pflücker JC. Three-dimensional architecture of the thymus is required to maintain delta-like expression necessary for inducing T cell development. J Immunol. 2006; 176:730–734. [PubMed: 16393955]
38. Arinobu Y, et al. Developmental checkpoints of the basophil/mast cell lineages in adult murine hematopoiesis. Proc Natl Acad Sci USA. 2005; 102:18105–18110. [PubMed: 16330751]
39. Babina M, et al. The transcription factor profile of human mast cells in comparison with monocytes and granulocytes. Cell Mol Life Sci. 2005; 62:214–226. [PubMed: 15666093]
40. Nishiyama C, et al. GATA-1 is required for expression of FcεRI on mast cells: analysis of mast cells derived from GATA-1 knockdown mouse bone marrow. Int Immunol. 2005; 17:847–856. [PubMed: 15967781]
41. Chen CC, Grimbaldeston MA, Tsai M, Weissman IL, Galli SJ. Identification of mast cell progenitors in adult mice. Proc Natl Acad Sci USA. 2005; 102:11408–11413. [PubMed: 16006518]
42. Iwasaki H, et al. The order of expression of transcription factors directs hierarchical specification of hematopoietic lineages. Genes Dev. 2006; 20:3010–3021. [PubMed: 17079688]
43. Laiosa CV, Stadtfeld M, Graf T. Determinants of lymphoid-myeloid lineage diversification. Annu Rev Immunol. 2006; 24:705–738. [PubMed: 16551264]
44. Laiosa CV, Stadtfeld M, Xie H, Andres-Aguayo L, Graf T. Reprogramming of committed T cell progenitors to macrophages and dendritic cells by C/EBPα and PU.1 transcription factors. Immunity. 2006; 25:731–744. [PubMed: 17088084]
45. Ishiko E, et al. Notch signals inhibit the development of erythroid/megakaryocytic cells by suppressing GATA-1 activity through the induction of HES1. J Biol Chem. 2005; 280:4929–4939. [PubMed: 15563463]
46. Tomita K, et al. The bHLH gene *Hes1* is essential for expansion of early T cell precursors. Genes Dev. 1999; 13:1203–1210. [PubMed: 10323870]
47. Nishimura S, et al. A GATA box in the GATA-1 gene hematopoietic enhancer is a critical element in the network of GATA factors and sites that regulate this gene. Mol Cell Biol. 2000; 20:713–723. [PubMed: 10611250]
48. Grass JA, et al. GATA-1-dependent transcriptional repression of GATA-2 via disruption of positive autoregulation and domain-wide chromatin remodeling. Proc Natl Acad Sci USA. 2003; 100:8811–8816. [PubMed: 12857954]

49. Zhou M, et al. Friend of GATA-1 represses GATA-3-dependent activity in $CD4^+$ T cells. J Exp Med. 2001; 194:1461–1471. [PubMed: 11714753]

50. Pai SY, et al. Critical roles for transcription factor GATA-3 in thymocyte development. Immunity. 2003; 19:863–875. [PubMed: 14670303]

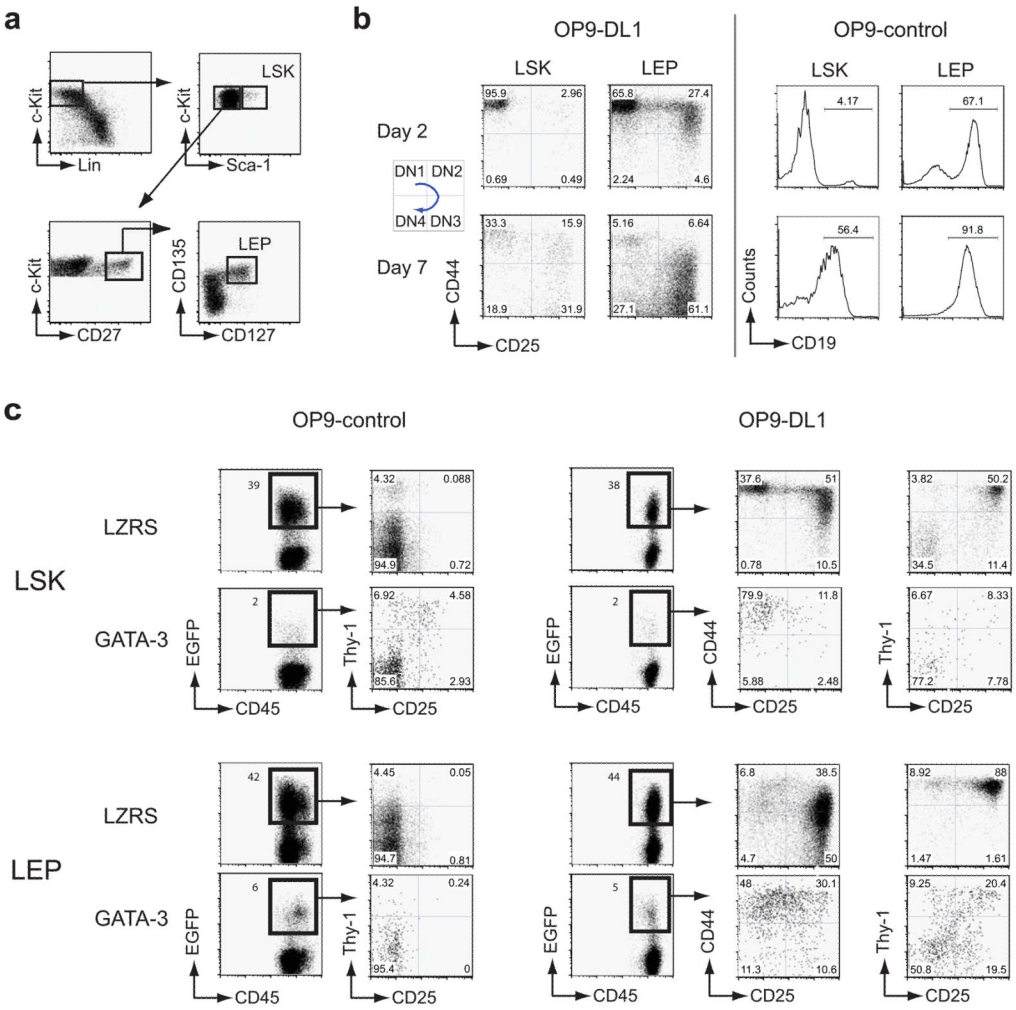

Figure 1. Inhibition of T-lineage specification of prethymic precursors by forced expression of GATA-3

(**a**) $Lin^{-}Sca1^{+}Kit^{+}$ (LSK) and Lymphoid Enriched Progenitor (LEP) cells were isolated from E14.5 BDF1 Lin− FL precursors as indicated. (**b**) FACs analysis comparing the differentiation kinetics of FL LSKs and LEPs after 2 or 7 days of OP9 coculture, showing pro-T cell development, based upon CD44 and CD25 expression, on OP9-DL stroma and B cell development, based on CD19 expression, on OP9-control stroma. (**c**) FACs analysis comparing Bcl2-tg LSK or LEP cells transduced with empty vector control (LZRS) and GATA-3 (GATA-3) cultured on OP9-control and OP9-DL1 cells. Transduced EGFP+ cells were gated and analyzed for the presence and relative percentages of T-lineage specified cells ($CD25^{+}Thy\text{-}1^{+}$) after 6 days in OP9-control cultures, or after 5 days in OP9-DL1 cultures. Results shown here and for sorted precursors without the Bcl2 transgene (Supplementary Fig. 1 online) are representative of at least 5 additional experiments using unsorted precursors, with and without the Bcl2 transgene.

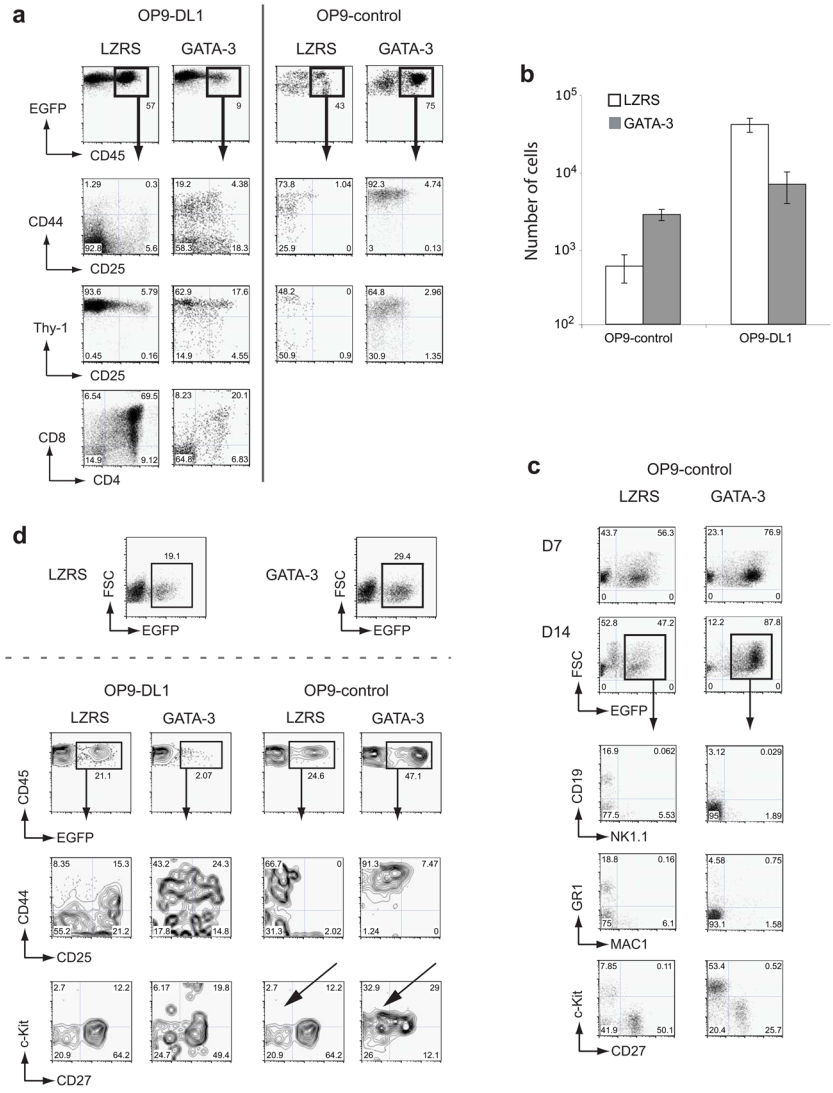

Figure 2. GATA-3 overexpression inhibits fetal thymocyte differentiation but enhances growth and non-T lineage diversion in the absence of Notch/DL1 signaling

(**a**) Development of C57BL/6 E14.5 fetal thymocytes after transduction with GATA-3 (GATA-3) or empty vector control (LZRS) following 5d of culture with OP9-DL1 or OP9-control stroma. FACs plots show the gated percentages of transduced $EGFP^+CD45^+$ cells and quadrant statistics for subsets within the $EGFP^+CD45^+$ gate. These results are representative of more than six independent experiments with fetal thymocytes from wildtype, Bcl2-transgenic, or Rag-deficient genotypes, except that viability losses were minimized with Bcl2-transgenic cells. (**b**) $EGFP^+$ cell recovery from LZRS-control or GATA-3 transduced fetal thymocytes split to parallel cultures in the absence (OP9-control) or presence (OP9-DL1) of Notch signaling, after 5 or 6 days in the indicated culture conditions. Results shown are geometric means from 2 representative experiments shown on a log scale; error bars indicate range. (**c**) Size and EGFP expression of LZRS or GATA-3 transduced BDF1 E14.5 fetal thymocytes after 1 or 2 weeks of culture on OP9-control, and surface expression of hematopoietic lineage markers by the $EGFP^+$ cells after 2 weeks of culture. (**d**) Responses of FL precursors precultured with OP9-DL1 before transduction with GATA-3. $c\text{-}Kit^+CD27^+Lin^-$ FL precursors were cocultured for 5 days on OP9-DL1, then transduced with LZRS or GATA-3 and replated on either OP9-control or OP9-DL1 cells for

6 additional days of coculture. FACs plots show FSC versus EGFP expression immediately after infection (upper panels) and after OP9-DL1 or OP9-control coculture (lower panels), with the phenotypes of the $EGFP^+$ cells. Arrows indicate c-Kit^{hi} $CD27^{lo}$ cells.

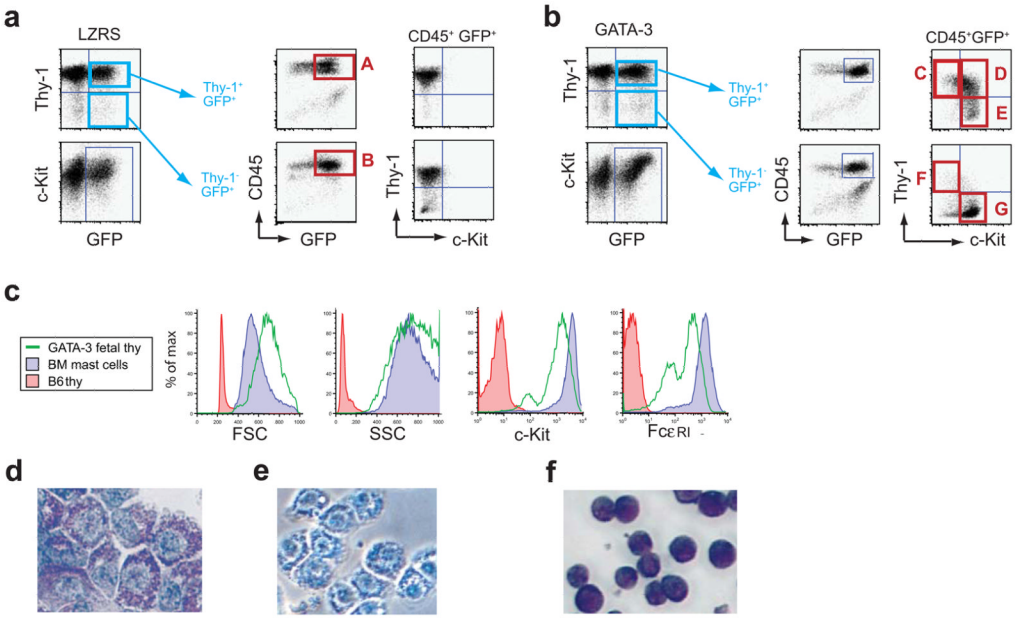

Figure 3. GATA-3 redirects Thy-1^{+} thymocytes to the mast cell lineage

Freshly isolated E14.5 C57BL/6 fetal thymocytes were transduced overnight with (**a**) LZRS and (**b**) GATA-3 and immediately sorted into GFP^{+}Thy-1^{+} and GFP^{+}Thy-1^{-} populations (blue boxes) followed by coculture with OP9-control cells. After 4 days cells were re-sorted based on expression of CD45, GFP, c-Kit and Thy-1 (red boxes, populations labeled A–G), which were subsequently characterized further in colony-forming assays (Table 1), mast cell culture conditions (panels c–d; & Table 1) and gene expression analysis (Fig 4c). LZRS controls, lacking c-Kit^{hi} cells, were sorted based upon CD45, GFP and Thy-1. Results shown are representative of two independent complete experiments. (**c**) Phenotypic analysis comparing GATA-3 diverted fetal thymocytes from population D (green lines), and BMMCs (blue solid histograms) after 2 wk culture in mast cell conditions, including IL-3 and SCF, with normal untreated adult thymocytes (red solid histograms). GATA-3 diverted thymocytes are more similar to BMMCs than normal thymocytes, displaying large size (FSC), and high granularity (SSC) and expressing high amounts of surface c-Kit and FcεRIα. (**d–e)** Histological staining of GATA-3 transduced fetal thymocytes (population D) cultured for 2 weeks in mast cell conditions and stained with (d) Giemsa and (e) a mast cell granule-specific stain, toluidine blue. (**f**) For comparison, normal fetal thymocytes stained with Giemsa.

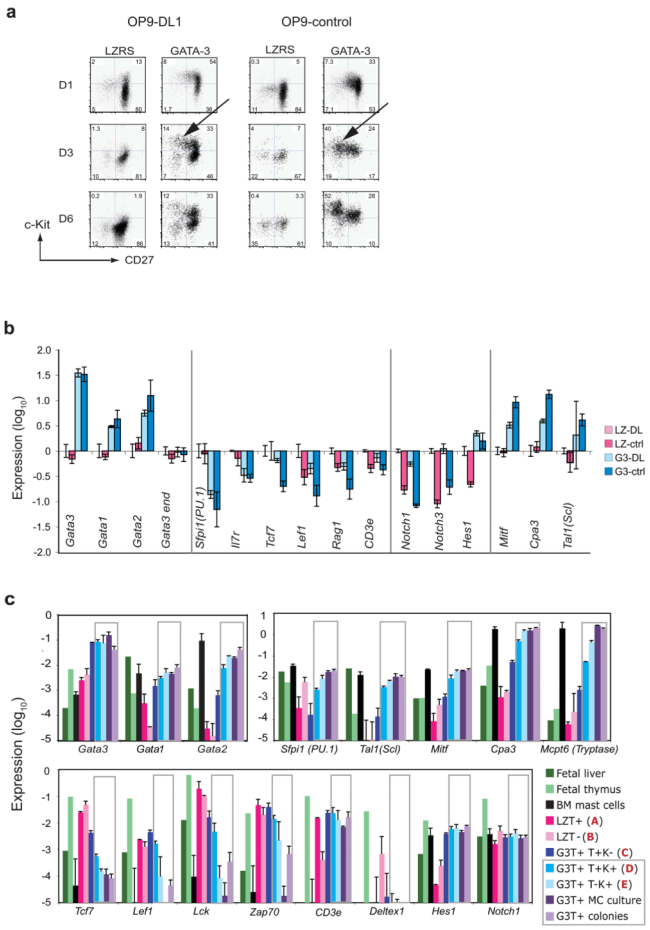

Figure 4. Kinetics of thymocyte reprogramming by GATA-3

(**a**) Kinetic analysis of differentiation of Bcl2-tg thymocytes transduced overnight with LZRS and GATA-3 and cocultured on OP9-control and OP9-DL1 cells with IL-7 and SCF. c-Kit and CD27 expression are shown for $CD45^{+}GFP^{+}$ cells, after 1, 3 and 6 days of culture. Arrows indicate c-Kit^{hi} $CD27^{lo}$ cells. Results are representative of two independent experiments. (**b**) Initial gene expression changes in the first day after GATA-3 transduction of Bcl2-tg thymocytes. Quantitative real-time RT-PCR analysis of gene expression in GFP^{+} $CD45^{+}$ transductants following 1 day of coculture with OP9-control or OP9-DL1 stroma (~40 hr overall). Data shown are the pooled average of the 4 samples derived from two independent experiments (see Supplementary Figure 4 online) with error bars indicating standard deviations. Results are normalized to β-actin and expressed on a log scale as ratios relative to LZRS controls with OP9-DL1. **(c)** Distinct gene expression effects of GATA-3 in transductants undergoing or resisting diversion. Gene expression analysis of C57BL/6 thymocyte populations 5 d after transduction from OP9-control coculture, as shown in Fig. 3a and b. LZRS transduced Thy-1^{+} and Thy-1^{-} cells (LZT^{+}, LZT^{-}: populations A, B) are compared with distinct subsets of GATA-3 transductants from Thy-1^{+} cells ($G3T^{+}$: populations C, D, E), and GATA-3 transduced Thy-1^{+} cells expanded in mast cell culture (MC culture) or in Methocult colony-forming assays (Colonies). T, Thy-1; K, Kit; gray boxes indicate GATA-3-diverted samples. Reference standards: FL (dark green), fetal thymocytes (light green), and BMMCs (black). Geometric means from two independent experiments are shown on a log scale in units relative to β-actin, with error bars indicating one standard deviation.

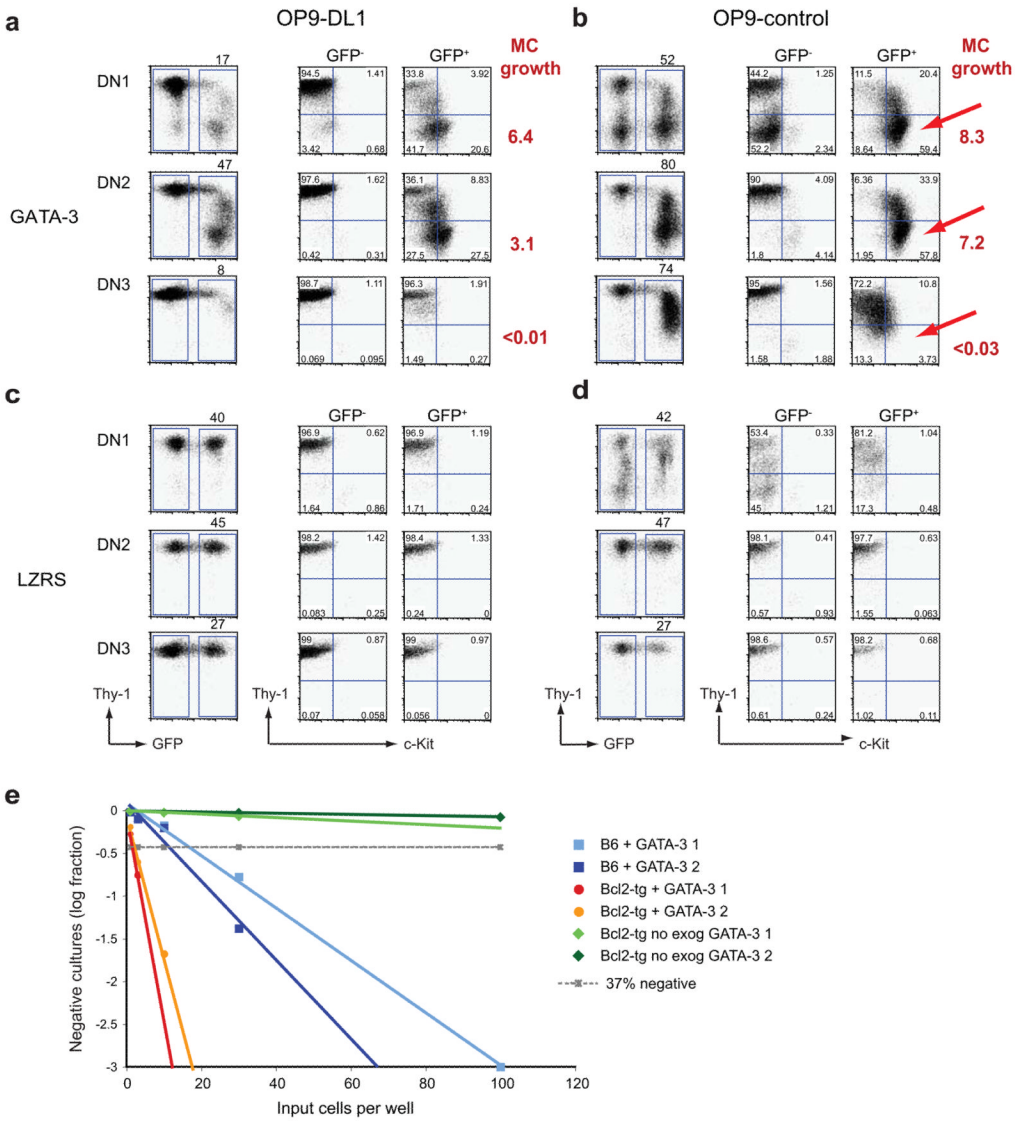

Figure 5. Developmental boundaries and frequency of GATA-3 mediated lineage diversion

(a–d) Development of sorted DN subsets. C57BL/6 DN1, DN2 and DN3 fetal thymocyte populations were sorted and then transduced overnight with GATA-3 or LZRS control. Samples were split for culture on OP9-DL1 or OP9-control stroma and analyzed after 5 days. Results are shown for GATA-3 transduced cells cocultured with OP9-DL1 (**a**) or OP9-control cells **(b)**, and LZRS transduced cells on OP9-DL1 **(c)** or OP9-control (**d**). All dot plots are gated for $CD45^+$, and sub-gated for GFP^- and GFP^+ cells. Red arrows indicate the $c\text{-}Kit^+Thy^-$ cell population. Numbers to the right of panels in (**a**) and (**b**) show the subsequent expansion (output/input cell numbers) of these populations 2 weeks after transfer to OP9-free culture with IL-3 and SCF. Results presented are one of two independent experiments with similar results. **(e)** Frequency of mast cell precursors in GATA-3 transduced DN2 fetal thymocytes after 10 day OP9-control coculture with IL-7, IL-3 and SCF. Limiting dilution analysis of GATA-3 transduced C57BL/6 DN2 (blue curves) and Bcl2-tg DN2 (orange curves) fetal thymocytes, showing the log fraction of cultures negative for mast cells. Untransduced Bcl2-tg DN2 fetal thymocytes (green curves) show very low, but detectable, mast cell development. Data are from 2 independent experiments.

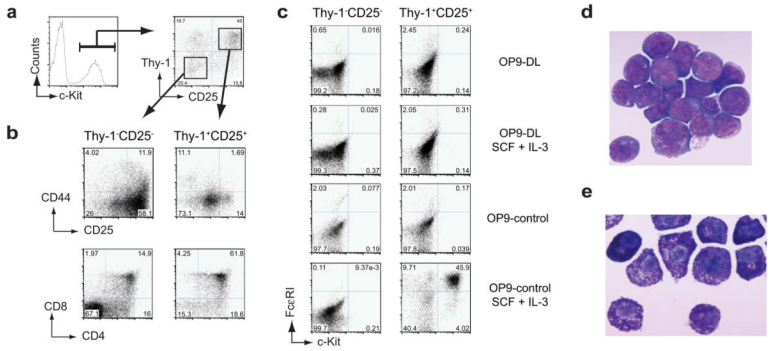

Figure 6. Mast-cell lineage potential of normal, unmanipulated fetal thymocytes

(**a**) c-Kit^{+}Thy-1^{-}CD25^{-} and c-Kit^{+}Thy-1^{+}CD25^{+} C57BL/6 E14.5 fetal thymocytes were sorted as shown. (**b**) T-cell developmental potential of c-Kit^{+}Thy-1^{-}CD25^{-} and c-Kit^{+}Thy-1^{+}CD25^{+} C57BL/6 fetal thymocytes cultured on OP9-DL1 stromal cells for 8 days. Both fractions include genuine T-cell precursors as both can give rise to CD4^{+}CD8^{+} double positive thymocytes on OP-DL1 with slightly different kinetics corresponding to their initial developmental stage. (**c**) Flow cytometric analysis of mast cell generation from c-Kit^{+}Thy-1^{-}CD25^{-} and c-Kit^{+}Thy-1^{+}CD25^{+} C57BL/6 fetal thymocytes on OP9-control and OP9-DL1 stromal cells in the presence of IL-7 and Flt3L, with or without the addition of SCF and IL-3. Dot plots show FcεRI and c-Kit surface expression on CD45^{+}-gated cells. (**d**) Wright-Giemsa staining of freshly isolated sorted c-Kit^{+}Thy-1^{+}CD25^{+} thymocytes from panel **a** that were used for culture experiments. (**e**) Wright-Giemsa staining of sorted FcεRI^{+}c-Kit^{+} cells, generated from c-Kit^{+}Thy-1^{+}CD25^{+} fetal thymocytes on OP9-control stromal cells, after 4 weeks of culture as shown in panel c. Data are representative of two independent experiments.

TABLE 1

GATA-3 MEDIATED GAIN OF GROWTH RESPONSE BY THYMOCYTES[1]

Infection	**LZRS**		**GATA-3**				
Phenotype before OP9-control coculture	**Thy$^-$**	**Thy$^+$**	**Thy$^-$**		**Thy$^+$**		
Phenotype before assay	**Thy$^-$ A**	**Thy$^+$ B**	**Thy$^+$ c-Kit$^-$ C**	**Thy$^+$ c-Kit$^+$ D**	**Thy$^+$ c-Kit$^-$ E**	**Thy$^+$ c-Kit$^+$ F**	**Thy$^-$ c-Kit$^+$ G**
Colony formation in Methocult: colony count							
Experiment 1[2]	1	2	nd	162	0	62	77
Experiment 2[3]	0	0	5	200	1	60	>120
Growth in mast-cell conditions: fold expansion relative to input							
Experiment 1	<1	<1	nd	130	<1	28	84
Experiment 2	<1	<1	<1	72	10	56	80

[1] Fetal thymocytes were retrovirally transduced with LZRS or GATA-3, sorted, cultured for 5 d on OP9-control, and re-sorted as shown in Fig. 3a, b. Samples of populations A—G were then cultured in cytokine-supplemented Methocult or grown in suspension with IL-3 + SCF as described in Methods.

[2] Inputs were 2.5×10^4 LZRS-transduced samples and 1×10^4 GATA-3 transduced samples.

[3] Inputs were 2.5×10^4 cells of each sample.