Tracing the first waves of lymphopoiesis in mice

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RAG1/GFP knock-in mice were used to precisely chart the emergence and expansion of cells that give rise to the immune system. Lymphopoietic cells detectable in stromal co-cultures arose as early as E8.5, i.e. prior to establishment of the circulation within the paraaortic splanchnopleura (P-Sp). These cells were Tie2^{+} RAG1^{−} CD34[Lo/−] Kit^{+} CD41^{−}. While yolk sac (YS) also contained lymphopoietic cells after E9.5, CD41^{+} YS cells from ⩽25-somite embryos produced myelo-erythroid cells but no lymphocytes. Notch receptor signaling directed P-Sp cells to T lymphocytes but did not confer lymphopoietic potential on YS cells. Thus, definitive hematopoiesis arises in at least two independent sites that differ in lymphopoietic potential. Expression of RAG1, the earliest known lymphoid event, first occurred around E10.5 within the embryos. RAG1/GFP^{+} cells appeared in the liver at E11.0 and progenitors with B and/or T lineage potential were enumerated at subsequent developmental stages.

KEY WORDS: Para-aortic splanchnopleura/aorta-gonad-mesonephros, Yolk sac, Thymus, Liver, Mouse

INTRODUCTION

The immune system is replenished throughout life by lymphocytes produced within the bone marrow (Nunez et al., 1996; Rossi et al., 2003). This process depends on stem cells defined in mice according to marker expression and long-term repopulating potential after transplantation to lethally irradiated recipients (Spangrude et al., 1988). Considerable information is available about the surface properties and gene expression of these stem cells, but many questions remain about their origin(s) during embryonic and fetal life (Dzierzak, 2003; Peault and Tavian, 2003; Phillips et al., 2000). Indeed, fetal stem cells and primitive lymphoid cells differ in a number of respects from their adult counterparts (Kincade et al., 2002; Yokota et al., 2003). Furthermore, there is evidence that hematopoietic stem cells (HSC) can arise from endothelial progenitors at multiple sites and stages of development (Oberlin et al., 2002). This raises the important possibility that independently arising stem cells yield distinct components of the immune system. Immune defense in the fetal/neonatal period might be provided by lymphocytes that are replaced from later emerging stem cells (Emambokus and Frampton, 2003; Mikkola et al., 2003b; Oberlin et al., 2002). Precise information about dynamics and characteristics of lymphoid populations would be helpful in resolving this important issue. The goals of this study were to localize and precisely time the appearance of progenitors for the first lymphocytes in embryonic life.

The relative importance of stem cells arising in intra-embryonic and extra-embryonic sites to definitive hematopoiesis has been extensively studied (Cumano et al., 1996; Cumano et al., 2001; Medvinsky and Dzierzak, 1996; Müller et al., 1994; Tavian et al., 2001). Primitive hematopoietic cells first appear within blood islands of the yolk sac (YS), but may be compromised with respect to

lymphopoiesis (Godin and Cumano, 2002; Metcalf and Moore, 1971; Palis and Yoder, 2001). Indeed, a four decade controversy regarding the origin of HSC endowed with lymphopoietic activity is still unsettled (Moore, 2004; Nishikawa et al., 1998). It appears likely that HSC that generate definitive red blood cells and lymphocytes emerge in association with the vasculature of the aorta-gonad-mesonephros (AGM) region, derived in part from the paraaortic splanchnopleura (P-Sp) (de Bruijn et al., 2002; Godin et al., 1999; Tavian et al., 1996; Tavian et al., 1999). Recent studies also show that the placenta may be a major site for expansion, if not production, of stem and progenitor cells (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). However, it has been argued that stem cells in the YS of E9 murine embryos are also capable of long-term multilineage reconstitution when transplanted into appropriately conditioned newborn mice (Yoder et al., 1997a; Yoder et al., 1997b). Therefore, doubt remains about the source of stem cells that account for the first lymphocytes. Moreover, the route connecting the first lymphopoietic cells to early lymphocyte progenitors (ELP) in liver or thymus is unclear.

Notch 1, but not Notch 2 is essential for conversion from primitive to definitive stages of hematopoiesis and the use of intra-embryonic rather than extra-embryonic sites (Kumano et al., 2003). It has also been reported that primitive hematopoietic cells can be converted to definitive ones by culture on AGM-derived stromal cells or by inducing HoxB4 gene expression (Kyba et al., 2002; Matsuoka et al., 2001). This suggests an important role for environmental cues such as Notch ligands that might be exclusive to intra-embryonic sites and we investigated that with respect to lymphopoiesis.

Elegant studies demonstrated that the thymus in avian embryos is populated and then repopulated by hematopoietic cells during discrete periods (Jotereau and Le Douarin, 1982). The thymus rudiment in mice is colonized at around embryonic day 11, but it is not clear if the first immigrants arise entirely or in part from intra-embryonic stem cells. In addition, previous studies have reached different conclusions about the degree to which the first thymus immigrants begin the T-lymphoid differentiation program before migrating to that site (Delassus and Cumano, 1996; Hattori et al., 1996; Ikawa et al., 2004; Péault et al., 1994). Nonetheless, there is near consensus regarding rapid commitment to the T-cell lineage on entry to the thymus because B lineage potential drops abruptly at E12-13 and is very rare at E14 (Péault et al., 1994; Schmitt et al.,

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2004). Fetal liver contains lymphoid progenitors with some degree of dedication to the T lymphoid lineage (Ema et al., 1998; Kawamoto et al., 2000; Yokota et al., 2003). Their relationship to the thymus also requires further study. The liver is a major site for expansion of long term repopulating stem cells (Ema and Nakauchi, 2000; Kumaravelu et al., 2002) and lymphocyte production in that organ has been characterized as being a synchronous wave (Strasser et al., 1989). This process has also been the subject of intense investigation, and the most numerous lymphocytes are dedicated to the B lineage (Yokota et al., 2003). A better understanding of lymphoid population dynamics in the fetal liver should be informative about many aspects of immune system development.

Analysis of RAG1/GFP knock-in mice revealed that RAG1 expression is a valid marker for the most primitive lymphoid progenitors in bone marrow and fetal liver (Igarashi et al., 2002; Yokota et al., 2003). This feature was exploited in the present study to learn how the very first populations of lymphocytes arise. We confirmed that the first lymphopoietic cells are contained within the angiopoietin receptor Tie2+ category of the P-Sp region and conclude that they must arise independently of the first definitive myelo-erythropoietic cells in the YS. Furthermore, we localized the earliest RAG1 expressing cells in the embryo proper. The RAG1 locus is activated in some thymus colonizers while still in the surrounding mesenchyme, and this event is followed by very rapid progression through the first stages of T lymphopoiesis. In addition to its role as a major producer of B lineage cells, the liver generates progenitors that can contribute to secondary waves of T lymphopoiesis. Thus, it is now possible to appreciate how unique lymphopoietic cells arise within the embryo and spawn the first components of the immune system.

MATERIALS AND METHODS
Mice and embryos
RAG1/GFP knock-in mice and their use in fetal studies have been described (Kuwata et al., 1999; Yokota et al., 2003). Mating homozygous male RAG1/GFP knock-in mice with wild-type C57BL/6 strain female mice generated heterozygous RAG1/GFP embryos with no contaminating maternal RAG1/GFP+ cells. In this study, the day of vaginal plug observation was considered as day 0.5 post-coitus (E0.5), and the staging of embryos was carefully confirmed by counting somite pairs.

Antibodies
FITC-conjugated anti-CD41 (MWReg30), anti-CD34 (RAM34) and anti-Kit (2B8) mAbs; PE-conjugated anti-CD19 (1D3), ant-TCRγδ (GL3), anti-Kit (2B8), anti-CD11b/Mac-1 (M1/70), anti-Sca1 (Ly6A/E; D7) and anti-CD25 mAbs; biotinylated anti-CD34 (RAM34) and anti-AA4.1 mAbs; allophycocyanin (APC)-conjugated anti-Kit (2B8), anti-TCRβ (H57-597) and anti-CD44 (1M7) mAbs; and purified anti-PECAM-1/CD31 (MEC13.3) mAb were all purchased from BD Pharmingen. PE-conjugated anti-Tie2 (TEK4) and APC-conjugated anti-CD45 (30-F11) mAbs were purchased from eBioscience. A PE-conjugated goat-anti-rat IgG Ab was purchased from Southern Biotechnology. A phycoerythrin-Texas Red tandem-conjugated streptavidin (PE-TR-streptavidin) was purchased from Caltag.

Immunohistochemistry
Embryos were accurately staged by counting somites and fixed in PBS containing 4% paraformaldehyde at 4°C overnight. Fixed embryos were embedded in PBS containing 7.5% gelatin, frozen in isopentane, cooled and then stored at –80°C. Cryostat sections (10 μm) were thawed and hydrated in PBS. As the intensity of GFP fluorescence decreases after fixation, the signal was amplified using an anti-GFP antibody. Slides were incubated overnight at 4°C with rabbit anti-GFP antibody (MBL, Japan), then washed in PBS and incubated at room temperature with Alexa Fluor 488-conjugated goat anti-rabbit antibody (Molecular Probes). Sections were mounted in Mowiol (Calbiochem) and examined under a DMR/HCS fluorescence microscope (Leica).

Flow cytometry
To determine the earliest timing of RAG1/GFP+ cell emergence in embryonic development, embryos were prepared between E6.5 and E11.5. Whole embryos or their dissected organs were mechanically minced, trypsinized, filtered through a nylon mesh of 70 μm (Cell Strainer; Falcon) into single cell suspensions and then evaluated by flow cytometry. The cells were stained with APC-conjugated anti-CD45 and 7-amino actinomycin D (7AAD, Calbiochem). Viable CD45+ cells were plotted on FL1-FL2 profiles without compensation. Gating for authentic GFP+ cells and exclusion of auto-fluorescent cells was performed as previously described (Yokota et al., 2003).

To examine the phenotype of hematopoietic cells in early embryos or those generated in organ cultures, tissues were dissociated by incubation with dispase II (Roche Diagnostics) for 20-30 minutes at 37°C and cell dissociation buffer (Invitrogen) for 30 minutes at 37°C followed by vigorous pipetting. The cells were resuspended in PBS containing 3% FCS and meshed to remove debris. After incubation with anti-FcR (2.4G2), the cells were stained with mAbs, suspended in 7AAD-containing buffer and analyzed.

In other experiments, sorted or cultured cells were incubated with anti-FcR and then stained with FITC-, PE-, APC-conjugated mAbs and a biotinylated Ab followed by PE-TR-streptavidin. All the flow cytometry analyses were performed with a FACScalibur using the Cellquest program (Becton Dickinson).

Organ cultures
The stromal cell line OP9 (a kind gift from Dr S.-I. Hayashi, Tottori University), MigR1(OP9-control) and murine Delta 1-like transduced OP9 (OP9-DL1) (Jaleco et al., 2001; Schmitt and Zúñiga-Pflücker, 2002; Witte et al., 1986) was maintained in α-MEM (Invitrogen, Carlsbad, CA) supplemented with 20% FCS (Hyclone, Logan, UT). Embryos were sterilely dissected under the microscope and the tissues (see Table 1) were placed on OP9 stromal cells prepared in six-well plates. The tissues were mechanically dissociated with 26-gauge needles and cultured in α-MEM supplemented with 20% FCS for 14 days by feeding with half medium changes every 2 days. When exogenous cytokines were used for T lineage differentiation, 5 ng/ml FLT3 ligand and 5 ng/ml IL7 (R&D systems, Minneapolis, MN) were added to the medium supplemented with 10% FCS and 50 μM 2-ME. At the end of culture, the tissues and cells were harvested from culture, treated with dispase II and cell dissociation buffer to make single cell suspensions, that were then subjected to flow cytometry analyses.

Cell sorting
YS or P-Sp/AGM regions obtained from E9.5 embryos of heterozygous RAG1/GFP knock-in mice were pooled and subjected to cell sorting. After the treatment with dispase II and cell dissociation buffer, the cells were suspended in PBS containing 3% FCS and incubated with anti-FcR. Then, the YS and P-Sp/AGM cells were stained with FITC-anti-CD41 and PE-anti-Kit mAbs, or with FITC-anti-Kit and PE-anti-Tie2, respectively. The cells were resuspended in 7AAD-containing buffer and subjected to sorting on a MoFlo (DakoCytomation).

E11.5-15.5 fetal livers were harvested and subjected to cell sorting as described previously (Yokota et al., 2003). In the first step, the cells were sorted into three populations (GFP−, GFP^Lo, GFP^Hi), according to levels of GFP expression. The cells were then fractionated according to Sca1 and Kit expression.

Methylcellulose culture
Two hundred and fifty cells of each sorted fraction (see Table 2) were cultured and evaluated for their colony forming activity in IMDM-based methylcellulose medium (Methocult GF3434, StemCell Technologies) as previously described (Yokota et al., 2003).

OP9 co-culture with sorted cells
The sorted YS or P-Sp/AGM cells were inoculated on OP9 stromal cells in six-well plates in the same way as the organ cultures described above. At day 5-6 of culture, hematopoietic colonies grown on or beneath OP9 cells were counted. They were also examined under a fluorescence-dissecting microscope to detect GFP expression during the culture period. After 14 days of culture, the cells were harvested and analyzed by flow cytometry. In

Table 1. Potential for RAG-1/GFP expression in OP9 stromal cell co-culture

Gestational age	Somites	Tested tissue	CD45$^+$/cultures*	GFP$^+$CD45$^+$/cultures†
E7.5	Presomite stage	Extra-embryonic area	3/3	0/3
		Embryonic area	3/3	0/3
E8.0-8.5	3-7	YS	18/19	1/19
		Caudal half	13/19	1/19
E8.5-9.0	8-12	YS	20/21	0/21
		P-Sp	11/21	3/21
		Heart (PB)	3/16	0/16
E9.0-9.5	13-20	YS	13/13	1/13
		P-Sp/AGM	11/13	6/13
		Neck	4/4	0/4
		Liver rudiment	4/4	0/4
		Heart (PB)	5/13	0/13
		Head	4/4	0/4
E9.5-10.0	21-29	YS	21/21	8/21
		AGM	20/20	17/20
		Neck	19/21	0/21
		Liver rudiment	20/21	4/21
		Heart (PB)	18/19	1/19
		Head	7/14	0/14
		Limb	5/5	0/5
E10.0-10.5	30-35	YS	9/9	5/9
		AGM	11/11	10/11
		Neck	11/11	3/11
		Liver rudiment	11/11	5/11
		Heart (PB)	7/7	4/7
		Limb	7/7	4/7
E11.5	45 or more	YS	8/8	8/8
		AGM	4/4	4/4
		Neck	8/8	6/8
		Liver	4/4	4/4
		Heart (PB)	3/3	3/3
		Limb	2/3	2/3

Embryonic tissues from the indicated stages were dissected and individually cultured on OP9 stromal cells in six-well plates. At 14 days of culture, tissues and cells were harvested and analyzed by flow cytometry.
*Hematopoietic cells were considered to be present in cultures when a minimum of 0.1% of CD45$^+$ cells was found among live mononuclear cells.
†RAG-1/GFP$^+$ CD45$^+$ cells constituted a very conspicuous proportion of the recovered cells whenever they were found, as illustrated in Fig. S1 (see supplementary material). A total of 96 embryos was individually classified according to somite pair numbers and tested in this culture system.

some experiments, progenitors sorted from E14.5 liver were cultured with OP9-control or OP9-DL1 for 2 weeks. At the end of culture, the cells were counted and analyzed by flow cytometry to determine the yield of T/B lymphocytes.

RESULTS
Spontaneous lymphopoiesis in early embryos
A flow cytometric method we previously developed for detection of extremely low levels of GFP in RAG1/GFP mice (see Materials and methods) was used in the present study to document the earliest expression of this marker in embryos. No RAG1/GFP$^+$ CD45$^+$ cells

were found prior to the E10.5 stage (Fig. 1A; data not shown). The yolk sac and placenta (Gekas et al., 2005; Melchers, 1979; Ottersbach and Dzierzak, 2005; Yoder et al., 1997a; Yoder et al., 1997b), as well as the P-Sp/AGM (de Bruijn et al., 2002; Godin et al., 1999; Tavian et al., 1996; Tavian et al., 1999) area within embryos, are sites known to be primary sites for initial generation and/or expansion of hematopoietic stem cells. Small numbers of GFP^{Lo} CD45$^+$ hematopoietic cells were present in the yolk sac and embryo bodies from E10.5, while they were also detectable in the placenta from E11.5 (Fig. 1A). Given the numbers of viable GFP^{Lo} CD45$^+$ cells, the embryo proper was the principal location of

Table 2. Clonal growth of the sorted E9.5 YS or P-Sp cells

Fraction	Methylcellulose culture			OP9 co-culture		
	CFU-G/M/GM	BFU-E	CFU-GEM(M)	Total cultured cells	Total colonies	GFP$^+$ colonies
YS CD41$^-$ c-kit$^+$	0	2	4	62,700	25	0
YS CD41$^+$ c-kit$^+$	49	86	32	26,150	922	0
YS CD41$^+$ c-kit$^-$	15	20	9	29,700	100	0
YS CD41$^-$ c-kit$^-$	0	0	0	416,000	0	0
P-Sp Tie2$^+$	0	0	0	24,890	21	12
P-Sp Tie2$^-$	0	0	0	713,000	0	0

YS cells or P-Sp cells were sorted from E9.5 embryos (17-25 somite pairs) according to the expression of CD41 and Kit, or Tie2, respectively. The sorted cells were cultured in methylcellulose media (total 250 cells of each fraction) or in OP9 stromal co-culture. The data shown represent the summary of three independent experiments using a total of 30 embryos.
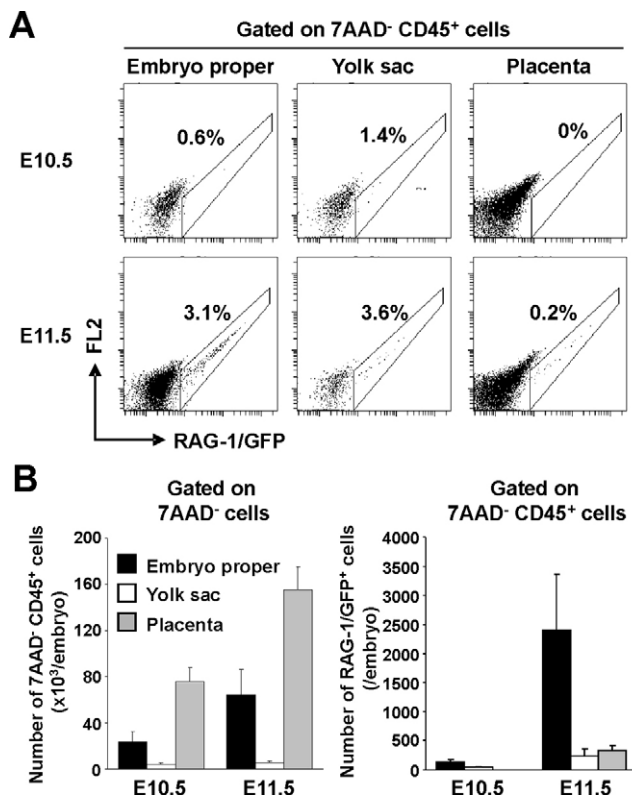
A

B

Fig. 1. The first lymphoid cells with spontaneous RAG1 expression arise within embryos on day 10.5. (**A**) Single cell suspensions were made from whole embryos or the indicated tissues (embryo proper, yolk sac, placenta), as described in the Materials and methods. Percentages indicate the frequencies of RAG1/GFP+ cells among viable 7AAD− CD45+ hematopoietic cells. The results are representative of three independent experiments. (**B**) The bar graphs depict numbers of 7AAD− CD45+ cells (left) or 7AAD− CD45+ RAG1/GFP+ cells (right) in the indicated tissues. The data represent mean values with standard deviations from four embryos and are representative of three independent experiments.

lymphoid progenitors and they expanded nearly 18-fold during this one day interval (Fig. 1B). The AGM region also contained a conspicuous GFP+ population at E11.5. However, this is unlikely to be an active site for lymphopoiesis, because percentages of GFP+ cells were not higher in this organ than in peripheral blood or other non-hematopoietic organs throughout the embryonic period (data not shown). We conclude that spontaneous RAG1/GFP expression initiates and expands in the embryo proper at around E10.5.

The first lymphopoietic cells develop mainly in the P-Sp/AGM region

Carefully dissected rudiments of individually staged embryos were next placed on monolayers of OP9 stromal cells in order to reveal their lympho-hematopoietic potential. CD45+ hematopoietic cells were produced in 2-week co-cultures of extra-embryonic, as well as intra-embryonic tissues, and even from pre-somite stages (Table 1). By contrast, functional progenitors that could efficiently generate viable CD45+ GFP+ cells only arose within the P-Sp area of E8.5-9.0 (8-12 somites) embryos. Indeed, most YS from E8.0-9.5 embryos gave rise to robust CD45+ populations that included Gr1+ granulocytes and TER119+ erythrocytes, but no RAG1/GFP+ cells (Table 1, see Fig. S1 in the supplementary material). It is noteworthy

that those YS-derived CD45+ populations included cells bearing CD45R/B220, which is commonly used as a marker for lymphoid cells (see Fig. S1 in the supplementary material). This demonstrates that lymphopoietic potential cannot be determined with this as the only marker. YS or liver rudiments harvested at a slightly later stage (E 9.5-10.0; 21-29 somites) also gave rise to CD45+ GFP+ cells. A majority (70-80%) of the CD45+ GFP+ cells recovered from 2-week cultures expressed CD45R/B220 and CD19, while some (~1%) displayed surface IgM (see Fig. S1 in the supplementary material).

Thus, primitive cells with the potential of generating the earliest lymphoid progenitors in stromal cell co-cultures are localized in the P-Sp area by E9.5. These lymphopoietic cells were easily detectable in the circulation, limbs, neck area and liver beyond E10.5, a stage when the circulation is known to be fully established (McGrath et al., 2003).

Primitive fetal cells that give rise to the immune system are responsive to Notch signals and can be directed to a T lineage lymphoid fate

Notch1 plays a key role in initiation of the definitive hematopoiesis program (Kumano et al., 2003) and we hypothesized that signaling via this receptor might induce lymphopoietic potential in extra-embryonic cells. However, neither RAG1/GFP nor B/T lineage markers were expressed when 15-20 somite YS cells were cultured with OP9 stromal cells transduced with the Delta 1 Notch ligand, OP9-DL1 (Schmitt and Zúñiga-Pflücker, 2002) (Fig. 2A). By contrast, P-Sp progenitors were very responsive to Notch signals and could be directed into the T lymphocyte lineage. They generated numerous RAG1/GFP+ CD45R/B220+ CD19+ cells on OP9-control cells (see Fig. S1 in the supplementary material), but B lymphopoiesis was completely arrested and conspicuous populations of TCR+ cells appeared in OP9-DL1 co-cultures (Fig. 2A). T lineage progression was also demonstrable with respect to CD4 and CD8 expression (data not shown). Given that TCRγδ+ cells normally constitute the first wave of lymphopoiesis in the fetal thymus (Havran and Allison, 1988), it was surprising to find that most lymphocytes produced in OP9-DL1 cultures expressed TCRβ (Fig. 2B). We then reduced concentrations of FCS from 20% to 10% and added the cytokines Flt3 ligand and IL7 to one group of cultures. Total cell recoveries were three- to fivefold higher, yields of TCRγδ+ cells were five- to eightfold more and TCRβ+ cells were still present under these conditions (Fig. 2B,C). Thus, primitive cells located within the P-Sp region of early embryos have the potential to make both major subsets of T lymphocytes, as well as B cells. Ligation of Notch receptors is sufficient to shift differentiation from the B lineage to the T lineage, and the ratio of TCRγδ+ to TCRαβ+ cell production is influenced by cytokines.

Characteristics of the earliest lymphopoietic progenitors

Flow cytometry experiments were then conducted to learn more about the properties of cells in early embryos endowed with the potential of generating lymphocytes in co-cultures. In particular, we used markers that have previously been used to characterize hemangioblasts, hemogenic endothelial cells and HSC. For example, the CD41 integrin is reportedly expressed by definitive HSC in YS (Corbel and Salaun, 2002; Mikkola et al., 2003a; Mitjavila-Garcia et al., 2002). We could easily resolve CD41+ cells in E9.5 (19-25 somites) YS and they could be further divided into subsets according to Kit or CD45 expression (Fig. 3A). CD41+ YS cells yielded myelo-erythroid cells in methylcellulose cultures (Table 2). Indeed, 922 and 100 hematopoietic colonies were
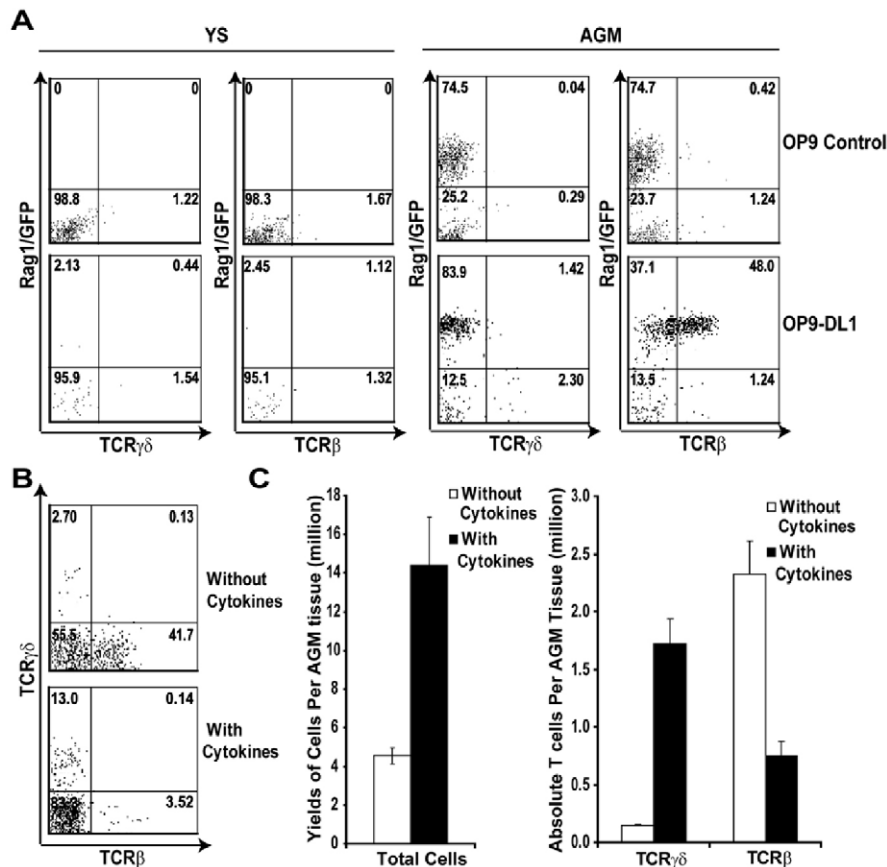
Fig. 2. Notch receptor ligation generates T cells from P-Sp/AGM, but not from YS of early embryos. (**A**) YS and P-Sp from E9.0-9.5 embryos (somites 15-20) were dissected and cultured on OP9 cells for 14 days without exogenous cytokines. The cultures were then stained for lymphocyte markers including TCRβ and TCRγδ. (**B**) E9.0-9.5 P-Sp were cultured on OP9-DL1 with or without additional cytokines for 14 days in parallel, the cultures were then stained for TCRγδ and TCRβ from comparison. (**C**) E9.0-9.5 P-Sp were cultured on OP9-DL1 with (black bars) or without additional cytokines (white bars) for 14 days in parallel. The total cell number per tissue (left) and T cells produced (right) are shown.

developed when a total of 26,150 CD41+ Kit+ cells or 29,700 CD41+ Kit− cells were cultured with OP9 stromal cells. However, no GFP+ or CD19+ cells were produced among the 1022 clones derived from CD41+ E9.5 YS cells (Table 2; see Fig. S2 in the supplementary material). This again indicates that YS initially lacks lymphopoietic cells demonstrable with the OP9-DL1 co-culture system.

In contrast to YS, CD41 was not clearly expressed in P-Sp/AGM tissue harvested from the same embryos (Fig. 3A). Threshold levels of VE-cadherin have been used to characterize hemogenic endothelium in early embryos (Nishikawa et al., 1998), and small numbers of RAG1/GFP+ fetal liver cells express this marker (Yokota et al., 2003). However, few distinctly positive cells were found in the E8.5-9.5 P-Sp. Therefore, we used the Tie2 angiopoietin receptor that has also been found on hemogenic endothelium at E10.5 (Hamaguchi et al., 1999). As shown in Fig. 3B, 1-2% of total mononuclear cells in E9.5 P-Sp/AGM clearly expressed this receptor and the Tie2+ cells were further characterized as CD34Lo/− Kit+.

The efficiency of generating CD45+ GFP+ CD19+ cells was reduced when the P-Sp was enzymatically dissociated and sorted before OP9 co-culture. However, 21 clones of hematopoietic cells developed when a total of 24,890 Tie2+ P-Sp cells were cultured with OP9 cells, and 12 out of the 21 clones contained RAG1/GFP+ CD45+ CD19+ cells (Table 2; see Fig. S2 in the supplementary material). No hematopoietic colonies developed from Tie2− cohorts. Flow cytometry analyses further verified that the colonies derived from YS cells in the OP9 co-culture contained no RAG1/GFP+ CD45+ CD19+ cells, while those from Tie2+ P-Sp cells generated robust B lymphocytes (Fig. 3C). As a more stringent test of lymphopoietic potential, stem cell factor, Flt3 ligand and IL7 were

added to the OP9 co-cultures. Confluent populations of approximately 80% CD19+ GFP+ lymphocytes were produced within 10 days from E9.5 Tie2+ P-Sp/AGM cells, while the Tie2− cohort contained no activity (T.Y., unpublished). Surprisingly, no myeloid colonies were grown from a total of 2,500 Tie2+ P-Sp/AGM cells in methylcellulose cultures in three experiments. Only small numbers of non-lymphoid cells arose when the same fraction was placed in stromal cell co-cultures. Thus, the most primitive of lymphopoietic cells have been localized in the P-Sp and defined as Tie2+ RAG1− CD34Lo/− Kit+ CD41−.

Initiation of lymphopoiesis in the fetal thymus

The thymus rudiment is devoid of lympho-hematopoietic potentiality until ~E10.5 when an influx of progenitors begins (Owen and Ritter, 1969). GFP+ cells, which were actually the first ones during embryonic development, became conspicuous both within and surrounding the thymus rudiment slightly later (Fig. 4A, left panel). At E12.5, lymphoid progenitors were more circumscribed and completely surrounded by epithelium in the E14.5 thymus (Fig. 4A, middle and right panels). Sufficient cells could be obtained from the E12.5 thymus for flow cytometry analysis. At that stage, more than 70% of the CD45+ mononuclear cells in the thymus expressed GFP and virtually all hematopoietic cells became GFP positive at E13 (Fig. 4B; T.Y., unpublished). Gating on those GFP+ cells revealed surprisingly homogeneous expression of Kit, CD34 and PECAM1/CD31 (Fig. 4C). Although E12.5 thymocytes were also uniformly CD4− CD8− CD44+ CD25− and Mac-1Lo, they rapidly differentiated (Fig. 4C,D; T.Y., unpublished). Indeed, nearly half of the cells, and especially ones with very high levels of GFP, had already progressed to the CD44+

$CD25^+$ DN2 stage in E13 embryos. These observations indicate that activation of the RAG1 locus by Kit^{Hi} $CD34^+$ $CD31^+$ $CD4^-$ $CD8^-$ $CD44^+$ $CD25^-$ Mac-1^{Lo} progenitors occurs within the vicinity of, or immediately after entry into, the thymus rudiment.

Population dynamics in the fetal liver

Organ culture data suggest that the fetal liver is colonized with hematopoietic cells as early as E9.0, a stage when its anlage emerges in the mesodermal septum transversum (Metcalf and Moore, 1971; Zaret, 1998). Thereafter, it is a major site for stem cell production and our goal was to chart accurately the appearance and expansion of lymphoid progenitors. RAG1 locus activation does not begin in this site until E11.0 (data not shown). This timing is important because it indicates that the first GFP^+ cells in liver could not contribute to ones emerging in the thymus at E10.5. At E11.5, one

Fig. 3. YS and P-Sp/AGM cells at E9.5 differ with respect to surface markers and lympho-hematopoietic potential. (**A**) YS and P-Sp/AGM cells from E9.5 embryos (somites 19-25) were stained with FITC-anti-CD41and APC-anti-Kit or anti-CD45. (**B**) E9.5 P-Sp/AGM cells were stained with PE-anti-Tie2 and FITC-anti-CD41, FITC-anti-CD34 or APC-Kit. (**C**) $CD41^+$ cells or $Tie2^+$ cells were sorted from E9.5 (somites 19-25) YS or P-Sp/AGM regions, respectively. The cells were co-cultured with OP9 stromal cells for 2 weeks, and then subjected to flow cytometry analysis after staining with PE-anti-CD19 and APC-anti-CD45. The data are representative of three independent cultures. In all these analyses, dead cells were excluded by staining with 7AAD.

liver contained ~800 RAG1/GFP^+ $CD45^{Hi}$ cells that corresponded to 3.8% of the $CD45^+$ cells (Fig. 5A). These earliest spontaneously arising GFP^+ cells were Kit^+ Mac-1^+ $AA4^{Lo}$ (Fig. 5A), properties that closely correspond to fetal stem cells (Godin and Cumano, 2002; Jordan et al., 1990; Morrison et al., 1995). This observation indicates that the first wave of hepatic lymphopoiesis may arise directly from immigrant stem cells.

We previously found that E14 liver could be resolved into 10 fractions on the basis of Kit, Sca1 and RAG1/GFP expression (Yokota et al., 2003). The OP9-DL1 co-culture system provided a means to simultaneously detect T and B lineage potential of these otherwise well characterized progenitors. RAG1/GFP^{Hi} progenitors that have high B potential in stromal cell free cultures (Yokota et al., 2003) were suppressed on OP9 stromal cells. OP9-DL1 completely blocked B lineage progression of RAG1/GFP^{Lo} cells and promoted them to T lymphopoiesis. The RAG1/GFP^{Lo} Kit^{Hi} $Sca1^+$ fraction was particularly effective in generating TCR^+ cells on OP9-DL1 (Fig. 5B). Surprisingly, the HSC containing RAG1$^-$ Kit^{Hi} $Sca1^+$ fraction did not produce large numbers of T-lineage cells in this model, although the same subset efficiently gave rise to $CD19^+$ cells on OP9. Furthermore, we know that the OP9-DL1 stromal cells support T lymphopoiesis from primitive P-Sp cells (Fig. 2). These results suggest that, while hepatic RAG1/GFP^{Lo} cells produce the first wave of B lymphopoiesis, they can also contribute to early T lineage development in thymus around E14.

Fig. 5C shows the kinetics of hepatic lymphopoietic progenitors resolved according to these parameters. The RAG1/GFP^{Lo} Kit^{Hi} $Sca1^+$ subset, that was the most potent with respect to T lineage in OP9-DL1 co-cultures peaked in number ($\sim6\times10^4$ liver) at E14.5. Restricted B lineage progenitors detectable in stromal cell free cultures are present in the GFP^{Hi} Kit^{Lo} $Sca1^-$ fraction and a majority (~75%) of them express CD19 at E14.5 (Yokota et al., 2003). Numbers of these cells increased dramatically from E14.5 (Fig. 5C). Indeed, there was logarithmic expansion of $CD19^+$ lymphocytes between 12.5 and 15.5 days of gestation.

Thus, hepatic lymphopoiesis begins around E11.0 and leads to explosive production of B lineage cells. Smaller numbers of cells with T lymphoid potential are present slightly later and might participate in secondary seeding of the thymus.

DISCUSSION

Our study focused on the first cells that give rise to progenitors with an active RAG1 locus, a characteristic closely related to B and T lymphopoiesis. The objective was to learn when and where lympho-hematopoietic cells initially appear, determine if they had distinctive surface markers and then document expansion of their lymphoid progeny. When considered together with temporal aspects of stem cell generation and establishment of the circulation, the information provides a basis for understanding how the immune system is built during early embryonic life.

Lymphopoietic cells first arise in an intra-embryonic site

The earliest hematopoietic cells appear within YS blood islands at around E 7.0 (pre-somite stage), followed by the P-Sp region at E9.5 (24-27 somites) (Medvinsky et al., 1993). Previous studies used RT-PCR to conclude that trace levels of RAG1 and RAG2 were detectable as early as E9.5, while active B lymphocyte precursors emerged in both liver and AGM at E11.5 (De Andrés et al., 2002; Marcos et al., 1997). It has been reported that the placenta contains B cell precursors and recent studies indicate that site harbors many stem/progenitor cells (Gekas et al., 2005; Ottersbach and Dzierzak,
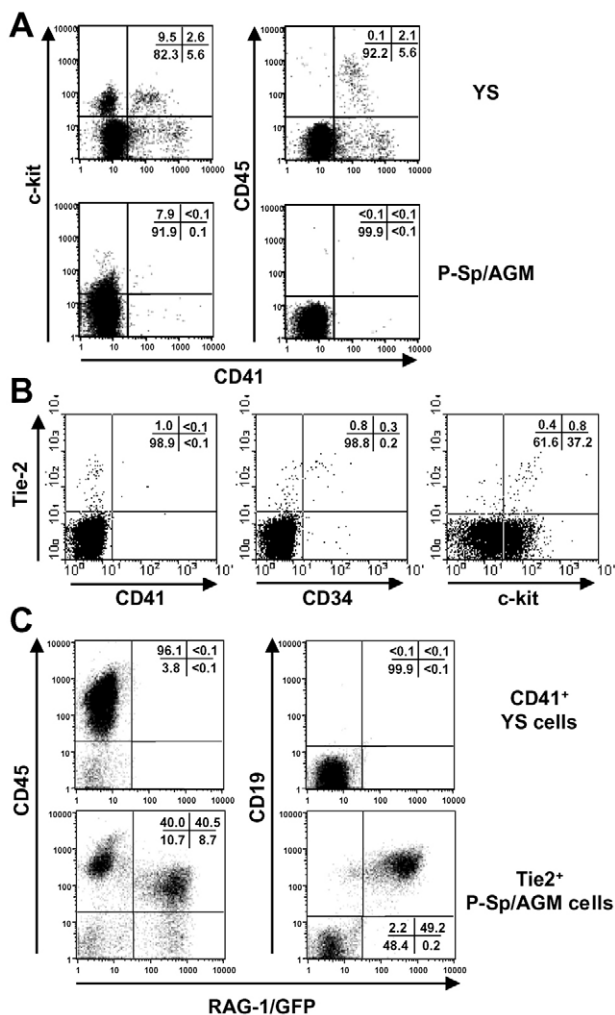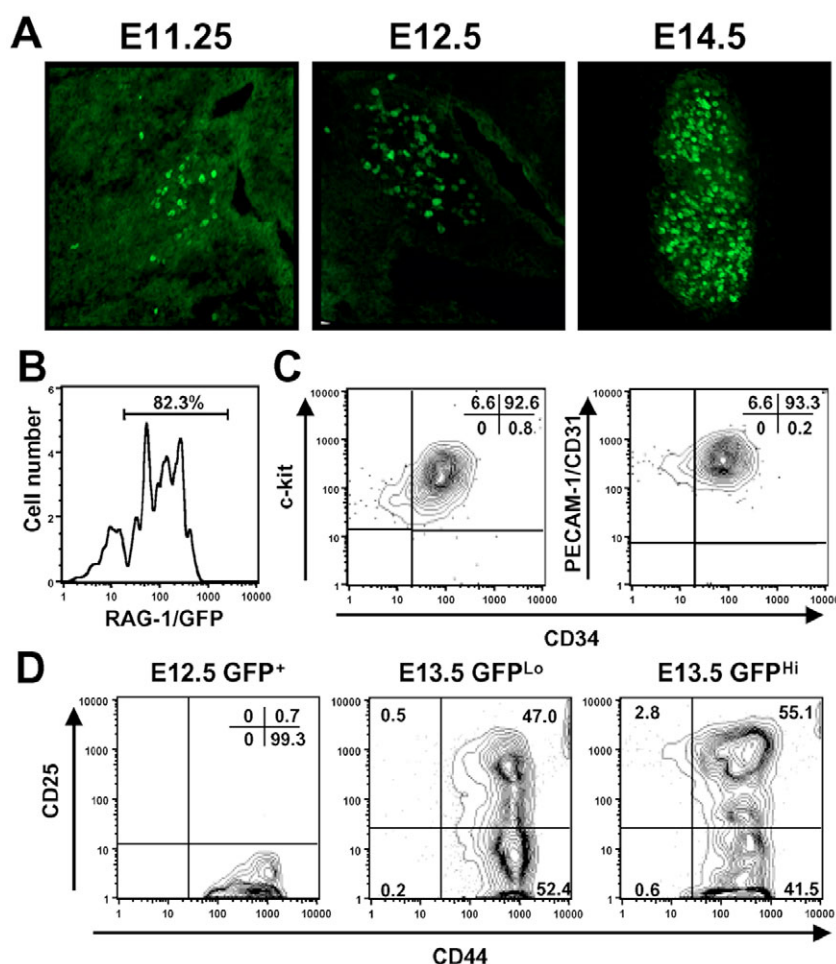
Fig. 4. Characterization of the first wave of T lymphopoiesis in the developing thymus. (**A**) Thymus rudiment sections from E11.25, E12.5 or E14.5 embryos were stained with an anti-GFP antibody. (**B**) Flow cytometry analysis showed that 82.3% of total CD45^{+} mononuclear cells in the E12.5 thymus expressed RAG1/GFP. The data is representative of three independent analyses. (**C**) GFP^{+} cells were sorted from the E12.5 thymus, and then stained with anti-PECAM-1/CD31 followed by APC-anti-Kit, biotinylated anti-CD34 and streptavidin-PE-TR. (**D**) GFP^{+} cells from E12.5 or E13.5 thymus were stained with PE-anti-CD25 and APC-anti CD44.

2005; Melchers, 1979). In our analysis, RAG1/GFP^{+} cells were undetectable before E10.5 and careful examination did not thereafter reveal a concentration within either the YS, P-Sp/AGM or placenta. Rather, the highest density was in the vicinity of the thymus rudiment around E11.0 and it appears unlikely the placenta is a primary lymphopoietic organ.

Co-culture on stromal cells can reveal the lymphoid potential of extremely primitive stem/progenitor cells in early embryos (Cumano et al., 1996; Nishikawa et al., 1998; Ogawa et al., 1988; Tavian et al., 2001). This powerful approach revealed that progenitors of RAG1/GFP^{+} cells were often present after E8.5 (eight somites) within the P-Sp, and after E9.5 (21 somites) in the YS. Although the vasculature and heart develop earlier, actual blood cell circulation was documented at ~E9.0 (McGrath et al., 2003). Many studies have shown that HSC bud from endothelial cells and are thus poised to be distributed from that time (de Bruijn et al., 2000; Oberlin et al., 2002; Tavian et al., 1999; Tavian et al., 1996). In addition, the vitelline artery connecting the AGM and YS is one site for de novo stem cell production (de Bruijn et al., 2000; Tavian et al., 1999). Determining the origin of stem/progenitor cells is therefore difficult at or beyond the E9.0 stage.

Our data supports previous work in indicating that lymphocytes derive from progenitors that are produced in an intra-embryonic site (Cumano et al., 1996; Cumano et al., 2001; Nishikawa et al., 1998). Although the P-Sp/AGM environment is apparently unable to initiate lymphoid gene expression, it generates cells that commit to that fate on migration to other organs. In that context, it is interesting

that co-culture with an AGM derived stromal cell line altered YS stem cells such that they could give rise to lymphocytes on transplantation (Matsuoka et al., 2001). It is possible that YS-derived progenitors migrate to the AGM and acquire lymphopoietic potential, but a lineage tracing experiment in *Xenopus* indicates that it is an unlikely event (Ciau-Uitz et al., 2000). In addition, we found differences in surface markers displayed by progenitors in the YS and P-Sp (Fig. 3). Notch1 is essential for establishment of intra-embryonic definitive hematopoiesis (Kumano et al., 2003) and might represent a cue for generation of lymphopoietic cells. However, we found that lymphocytes were not produced when E8.5 YS was held on Delta1 transduced OP9 stromal cells.

CD41 has been reported to be a marker for definitive hematopoiesis in YS (Mikkola et al., 2003a) and we thought that display of this marker would provide evidence for a relationship to the first lymphopoietic cells. However, we found that while the non-lymphoid progenitors that develop in OP9 co-cultures were CD41^{+}, sorting for this characteristic from the YS of ≤25-somite embryos did not enrich primitive cells with the potential to express RAG1/GFP. Cumano and colleagues recently published a description of E10.5 AGM, where extremely low levels of CD41 were present on multipotent progenitors (Bertrand et al., 2005). Their interesting study suggests that many of the lymphopoietic cells we have characterized at E9.5 could be located in sub-aortic patches as mesodermal ancestors of HSC. However, surface levels of CD41 at that early stage did not permit its use in manipulation of intra-embryonic hematopoietic cells.
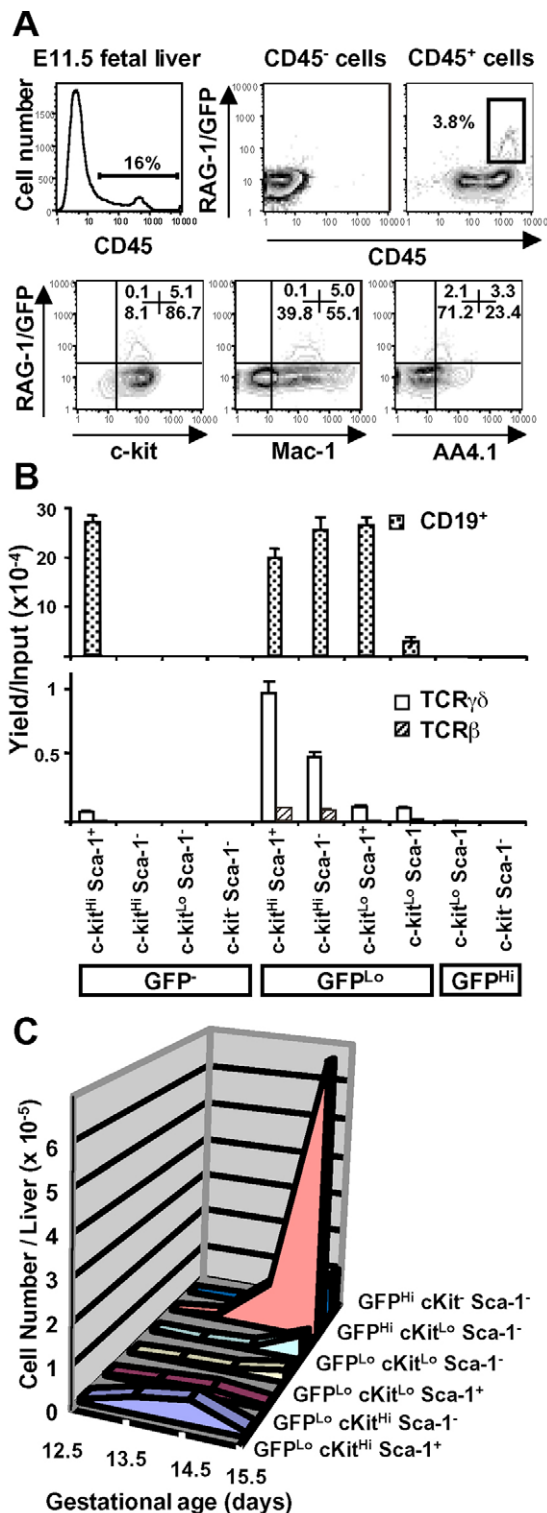
DEVELOPMENT

A

E11.5 fetal liver

CD45⁻ cells   CD45⁺ cells

3.8%

16%

B

C

Gestational age (days)

Fig. 5. Characteristics of the most primitive lymphoid progenitors and their progeny in the fetal liver. (**A**) Fetal liver cells from E11.5 embryos were stained with APC-anti-CD45 (upper, left panel) and sorted into CD45⁻ and CD45⁺ fractions (upper, middle and right panels). The sorted CD45⁺ cells were then stained with PE-anti-Kit, PE-anti-Mac-1 or biotinylated anti-AA4.1 followed by PE-TR-streptavidin (lower panels). Flow cytometry results are shown illustrating the expression of these markers on RAG-1/GFP⁺ progenitors. (**B**) Progenitors from E14.5 fetal liver were sorted on the basis of RAG1/GFP, Kit and Sca1 (Yokota et al., 2003) and then cultured on OP9-control or OP9-DL1 for 14 days. Yields of CD19⁺ B cells (dotted bars in the top panel), TCRγδ⁺ (white bars, lower) or TCRβ⁺ (hatched bars, lower) T cells per input progenitors are shown. (**C**) All lymphoid progenitor containing subsets of fetal liver at the indicated gestational stages were resolved. Percentages of each cell type were multiplied by cell yields to obtain absolute numbers per organ.

reported that multipotent long-term reconstituting HSC dominantly emerge in P-Sp/AGM, although this occurs after E10.0 (30 somites) in mice (Cumano et al., 2001; Medvinsky and Dzierzak, 1996; Müller et al., 1994).

It will be interesting to explore the relationship of Tie2⁺ progenitors in E9.5 P-Sp/AGM to subsequent waves of lymphopoiesis and learn if they are involved in replenishment of the immune system throughout life. Fate-mapping experiments with additional markers could be used to address this important issue. In this context, it is interesting that CD41 only marks a small subset of lympho-hematopoietic cells after the fetal/neonatal stage (Emambokus and Frampton, 2003). We conclude that CD41⁺ YS cells lacking lymphoid potential produce the first wave of definitive hematopoiesis and are subsequently replaced by Tie2⁺ cells that initially generate the immune system.

RAG1 gene expression in the vicinity of the thymus rudiment

The first wave of T lymphopoiesis, characterized by TCR γ/δ receptor expression (Havran and Allison, 1988), probably derives from P-Sp/AGM progenitors or ones that leave the liver before substantial progression in any lymphoid lineage. In the avian embryo where the liver is not hematopoietic, the first T progenitors originate in paraaortic mesoderm and initially colonize the thymus via blood delivery (Dunon et al., 1999). There has long been a controversy regarding the potential of the earliest thymus immigrants (Delassus and Cumano, 1996; Hattori et al., 1996; Ikawa et al., 2004; Péault et al., 1994) Some groups thought they were multipotent, whereas others insisted that commitment to T lineage was determined at a pre-thymic stage. Those conclusions might not be mutually exclusive, as recently discussed regarding T-lymphopoiesis in adults (Petrie and Kincade, 2005). We show here that the RAG1 locus is activated even before cells are fully encapsulated within the E11.25 thymus, although the cells retain surface markers corresponding to HSC (Fig. 4) (Suniara et al., 1999). Transcription of the ikaros gene was also detectable in progenitors before they entered the thymic epithelial region (Itoi et al., 2001). Thymic mesenchymal cells, which still surround the thymus anlage at E11.5, may create an environment that is crucial for early T cell development (Suniara et al., 2000), or the process could be initiated in fetal liver progenitors (Harman et al., 2005; Ikawa et al., 2004). As fetal thymocytes normally use γ/δ T cell receptors, it was surprising to find that TCRαβ lymphocytes predominated in co-cultures initiated with P-Sp/AGM cells.

It has previously been shown that the Tie2 angiopoietin receptor marked a discrete population of cells in E9.5 connecting arteries and E10.5 AGM (Hamaguchi et al., 1999; Takakura et al., 1998). We found Tie2⁺ Kit⁺ CD34^{Lo/−} cells in the E9.5 P-Sp/AGM and showed that they could give rise to RAG1/GFP⁺ CD19⁺ lymphocytes in stromal cell co-cultures. These findings confirm that this antigen marks the first lymphopoietic cells in embryonic life. However, it is still unclear whether those cells correspond to hemogenic endothelium, 'definitive' HSC or other cell types. Two groups

However, addition of IL7 to the cultures enhanced production of TCRγδ$^+$ cells. Cytokine concentrations in early fetal versus later thymuses might account in part for the TCR rearrangement and selection events needed to generate these two major types of T lymphocytes (Kang and Der, 2004; Zamisch et al., 2005). Appropriate and timely delivery of Notch signals may also govern the α/β versus γ/δ choice (Washburn et al., 1997; Wolfer et al., 2002). It is only important to stress here that the most primitive lymphopoietic cells thus far identified have the potential to generate both.

The fetal liver provides an environment for expansion of B lineage cells and generation of secondary thymic colonizers

Several significant points emerged from our analysis of lymphoid progenitors in liver. RAG1/GFP^{Hi} subsets of lymphoid progenitors in the developing liver accumulated in concert with organ size. Many studies have documented tremendous expansion of B-lineage cells in that site (Strasser et al., 1989). The conspicuous GFP^{Hi} Kit^{Lo} Sca1$^-$ subset expresses little GATA3 and rapidly generates CD19$^+$ cells in stromal cell free cultures (Yokota et al., 2003). However, all GFP$^+$ subsets are in cycle (20-30% in S+G2+M) and rapidly incorporate BrdU (R. Pelayo, unpublished). Therefore, there must be rapid progression through differentiation stages and/or export to other organs. The GFP^{Lo} cells in E13-14 liver would seem to be good candidates for secondary thymus seeding and some of them might represent the Kit^{Lo} progenitors of TCR α/β$^+$ T cells present in blood at that stage (Carlyle and Zúñiga-Pflücker, 1998; Rodewald et al., 1994).

There has been substantial controversy concerning the presence of lymphoid restricted progenitors in fetal tissues. For example, a series of impressive studies used a fetal thymic organ culture system to conclude that single T/B restricted cells without myelo-erythroid potential do not exist (Katsura, 2002). We have recently found that non-lymphoid options are sharply downregulated with activation of the RAG1 locus in fetal liver cells and RAG1^{Lo} Kit^{lo} subsets had the most potential for T lineage differentiation in a fetal thymus reaggregate culture containing IL3 (Yokota et al., 2003). We now report that the best subsets for T cell production in OP9-DL1 co-cultures expressed Kit at high density (Fig. 5B). A RAG1^{Lo} Kit^{lo} Sca1$^+$ fraction was an efficient source of CD19$^+$ B lineage cells when cultured with OP9 stromal cells and, although this activity was blocked by expression of Delta1, they generated only small numbers of TCR$^+$ lymphocytes. These observations highlight the importance of experimental conditions in determining how differentiation potential is assessed. In addition, we stress that production of B cells from non-committed progenitors would not be possible within a micro-environmental niche that is ideal for T lymphopoiesis (Schmitt and Zúñiga-Pflücker, 2002). Two RAG1^{Lo} Kit^{Hi} fractions of fetal liver are rich in progenitors for the two lymphoid lineages, but it would be difficult to ascertain if individual ones are B-only, T-only or bi-potent. That would require expansion of individual cells without introducing bias and then separately testing aliquots of progeny cells under ideal conditions for each lineage.

Concluding remarks

Our data support and extend previous studies (Godin and Cumano, 2002; Tavian et al., 2001) in suggesting that the P-Sp/AGM first generates lymphopoietic cells. Although adult-type myelo-erythroid progenitors may emerge from a CD41$^+$ population found in the YS, that organ is unlikely to be essential for building the immune system.

Indeed, lymphoid progenitors previously found in the YS (Paige et al., 1979; Weissman et al., 1977) could have arisen in the P-Sp and migrated to extra-embryonic tissues via the circulation. Lymphopoietic potential appears independently of the YS and is associated with a Tie2$^+$ Kit$^+$ CD34$^{Lo/-}$ CD41$^-$ subset that may arise even before conventional stem cells. Activation of the RAG1 locus is one of the earliest events in lymphopoiesis and that first occurs in an intra-embryonic site.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/10/2041/DC1

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