**Int. J. Dev. Biol. 49: 279-284 (2005)**
doi: 10.1387/ijdb.041936cc

# αIIb integrin, a novel marker for hemopoietic progenitor cells

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**ABSTRACT Integrin αIIbβ3 (abbreviated as αIIb), also known as GPIIb-IIIa or CD41/CD61, is a cell adhesion molecule expressed on cells belonging to the megakaryocytic lineage. Aiming to identify new markers of hemopoietic progenitor cells (HPC), we undertook a developmental study of this molecule since it remains controversial if this integrin is expressed by various progenitors. We reported the expression pattern of two integrins, in both of which the β3 chain is present, respectively associated with αV and α IIb in the chick embryo. While at E3.5, the earliest time at which these integrins can be detected, αVβ3 becomes expressed by endothelial cells in the aorta (and only in the aorta), αIIbβ3 becomes detected in the well-defined intra-aortic clusters made up of HPC. The latter were found to be multilineage progenitors when sorted for αIIb expression and analyzed by means of clonogenic assays. In mice also, αIIb is expressed in the intra-embryonic site of HPC generation, the intra-arterial clusters in the embryo proper, as well as in sites where HPC migrate. Finally we provided the first evidence in two species that multipotent HPC expressing αIIb are able to differentiate not only into cells of the erythroid and myeloid lineages but also into lymphocytes. These cell populations actually coexpress αIIb and c-Kit. These data establish αIIb as a novel marker for HPC, which appears at very early stages in the embryo. Capitalizing on this finding, other investigators confirmed it and suggested that αIIb plays a role in regulating hematopoietic development.**

KEY WORDS: *integrin, embryo, fetal liver, bone marrow, hemopoiesis*

## Introduction

Several markers such as Sca-1, Thy-1, c-Kit, CD34 and AA4.1 are often used to enrich hemopoietic progenitor cells (HPC) (Spangrude *et al.,* 1991). These markers are however differently expressed according to the embryonic, fetal or adult source of HPC. Accurate identification of HPC, which requires combination of markers, would benefit from the discovery of more specific surface molecules.

The β3 subfamily of integrins comprises αIIbβ3 and αVβ3 which are both expressed on megakaryocytic/thrombocytic cells (Hynes, 1992). The αIIb molecule (also called GPIIb or CD41) associates with β3 (GPIIIa or CD61) to form integrin αIIbβ3, a complex that labels the whole megakaryocytic (Mk) differentiation pathway. This integrin has been extensively studied for the fundamental role it plays in the function of Mk, mediating cell-substratum adhesion and platelet aggregation (Naik and Parise, 1997). While αIIb is expressed throughout differentiation by cells of the Mk lineage, some evidence has suggested that it is also present on other HPC. Using antibody inhibition assays on mouse cells, Berridge *et al.* (1985) reported the expression of this integrin on myeloid progenitors and on CFU-S from bone marrow (BM). By means of a conditional knock out, mice have been generated in which a thymidine kinase gene was placed under the control of the αIIb promoter. Upon ganciclovir administration, when all thymidine kinase expressing cells were eradicated, the growth of BM myeloid and erythroid progenitors as well as that of mixed progenitors (CFU-Mix) was dramatically reduced (Tronik-Le Roux *et al.,* 1995; Tropel *et al.,* 1997). In human, different types of HPC, including CFU-Mix, were found within the αIIb positive cell population from BM and cord blood (Fraser *et al.,* 1986; Murray *et al.,* 1996).

However the presence of αIIb on progenitors other than megakaryocytic has been a controversial issue. We decided to address this question in the chicken embryo which can be accurately staged, allows precise localization of emerging HPC at E3.5-4 in intra-aortic foci and at E6 in the para-aortic mensenchyme (Dieterlen-Lièvre and Martin, 1981). T lymphoid differentiation can also be

*Abbreviations used in this paper:* BM, bone marrow; EC, endothelial cell; En, n days of incubation or gestation; FL, fetal liver; HC, hemopoietic cell; HPC, hemopoietic progenitor cell; Mab, monoclonal antibody; Mk, megakaryocyte.

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0214-6282/2005/$25.00
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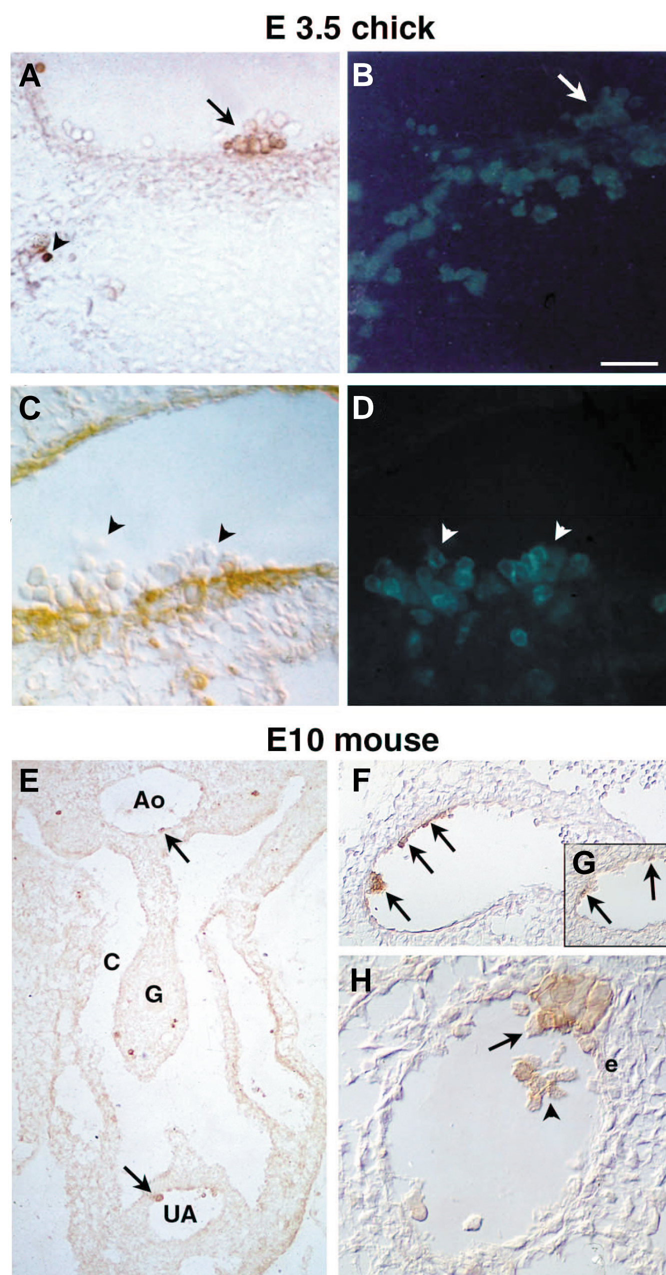


**Fig. 1. αIIbβ3 expression in the early intraembryonic hemopoietic sites. (A-D)** *Transverse sections of E3.5-4 chick embryo.* **(A,B)** *Double immunostaining with the anti-αIIbβ3 Mab, 11C3 (A), and the anti-CD45 Mab, HISC7 (B). Intra-aortic clusters (arrows) are αIIbβ3+ and a few positive cells are scattered in the mesenchyme beneath the aortic endothelium (arrowhead).* **(C,D)** *Double immunostaining with the anti-αVβ3 Mab, LM609 (C), and the anti-CD45 Mab, HISC7 (D). The endothelium of the dorsal aorta is αVβ3+ while intra-aortic clusters (arrowheads) are αVβ3-.* **(E-H)** *E10 mouse embryos.* **(E)** *αIIbβ3 positive clusters polarized on the floor of the dorsal aorta (arrowhead) and on one of the umbilical arteries (arrow).* **(F)** *Umbilical artery, showing αIIbβ3 positive clusters (arrows).* **(G)** *Umbilical artery from a consecutive section of (F), showing CD45low clusters (arrows).* **(H)** *Umbilical artery with a αIIbβ3 positive cluster that appears to be emerging from the endothelium (arrow). Some αIIbβ3 positive cells are present in the lumen of the vessel (arrowhead). Ao, aorta; C, coelomic cavity; G, gut; e, endothelium; UA, umbilical artery.*

monitored since access to the embryonic and adult thymus is easy; furthermoreT cell differentiation can be readily followed after intrathymic injection of progenitors, using two congenic strains of chicken. The chicken model was thus selected to characterize the differentiation potential of αIIbβ3+ HSC further. We demonstrated that, at E3.5-4, αIIb can be detected on HPC of the intra-aortic clusters and on embryonic and adult BM progenitors belonging not only to the erythro-myeloid lineages but also to the T lymphoid lineage (Ody *et al.,* 1999).

It appeared important to extend this demonstration to mice where HSC potential, i.e., the ability of αIIb+ sorted cells to long-term repopulate lethally irradiated host and to self-renew, was determined (Corbel and Salaün, 2002).

The other integrin in the β3 family, αVβ3, was known to be expressed by endothelial cells (EC). But its developmental expression pattern was not established previously, either in birds or in other species. Therefore one of us analyzed it *in situ* in the chick embryo using the anti-human αVβ3 Mab, LM609, which cross-reacts with the chicken molecule (Corbel, 2002). The main findings will be reviewed here.

## αIIb expression by intraembryonic hemopoietic progenitors

### *Chicken*

We characterized previously a monoclonal antibody (Mab), 11C3, directed against chicken αIIbβ3 (Lacoste-Eleaume *et al.,* 1994). In the embryo proper, αIIb was first expressed at E3.5-4 on cells located on the floor of the aorta, i.e., the typical intra-aortic clusters (Fig.1A). In this site the cells expressing αIIb are clearly not thrombocytes. These αIIb+ cells co-expressed the pan-leucocyte marker CD45 (Fig.1B). Interestingly αIIb expression was restricted to hemopoietic cells (HC), while endothelial cells were not stained. While αIIbβ3 is expressed only by HC, αVβ3 is expressed by both HC and endothelial cells and also osteoclasts and some metastatic melanomas.

On day 2 of development, αVβ3 expression was restricted to very rare cells in the blood stream, the embryo proper and the yolk sac blood islands, which are probably cells of the thrombocytic lineage. On day 3 it became detectable on EC of the dorsal aorta and was restricted to this blood vessel. Interestingly, αVβ3 was absent from the intra-aortic hemopoietic clusters when they developed. The endothelium underlying intra-embryonic hemopoietic clusters expressed this integrin. This is illustrated in Figure 1C-D. Therefore, the two β3 integrins are differentially expressed at E3.5-4, αVβ3 being a marker for endothelial cells while αIIbβ3 is a marker for HPC and uniquely labels the intraaortic clusters.

TABLE 1

**NUMBER OF MYELOID AND ERYTHROID PROGENITOR CELLS DEVELOPING FROM αIIbβ3 POSITIVE INTRA-AORTIC CELLS FROM E3.5-4 CHICK EMBRYOS**

| Culture conditions | αIIbβ3 sorted cells | Colony number / 10 000 cells |
|---|---|---|
| Myeloid | + | 573 ± 98 |
| | - | 33 ± 31 |
| Erythroid | + | 701 ± 205 |
| | - | 16 ± 6 |

***Mouse***

In view of our findings in birds, it was of interest to determine whether αIIb was also expressed by multilineage progenitors cells in the mouse embryo and fetus and whether could be considered as a feature common to birds and mammals.
Murine HPC are found in several sites, the yolk sac, the umbilical and vitelline arteries, the para-aortic splanchnopleura, the aorta/genital ridges/mesonephros (AGM) and the fetal liver (FL), the latter remaining the predominant hemopoietic site throughout fetal life (Moore and Metcalf, 1970; Cumano *et al.,* 1996; Medvinsky and Dzierzak, 1996; Yoder *et al.,* 1997; North *et al.,* 1999; de Bruijn *et al.,* 2000). αIIb expression was analyzed by *in situ* immunohistochemistry on sections. In the embryo proper at E10, αIIb was expressed in the clusters of hemopoietic cells associated with the wall of arteries, dorsal aorta and umbilical arteries (Fig.1E-H) and in the blood islands of the yolk sac. As in the avian model, the hemopoietic clusters expressed both αIIb and CD45 but with different antigenic intensities.

At E10.5 and E11.5, the FL, when becoming hemopoietic, contained numerous αIIb-positive cells. Characteristical polynucleated Mk were found at later stages of FL development.

## Analysis of the hemopoietic potential of αIIb sorted cell populations

***Chicken***

*Myeloid potential of intra-aortic clusters*

E3.5 aortic area cells were sorted for the expression of αIIβ3 and cultured in semi-solid medium in conditions appropriate to evaluate their hemopoietic progenitor potentialities according to Cormier and Dieterlen-Lièvre (1988). FACS analysis showed that 7%±2% of E3.5-4 aortic cells were αIIb+. Most of the colonies developing under myeloid and erythroid conditions grew from this αIIb-positive cell population (Table 1). Selection by αIIb led to a 20-fold enrichment in hemopoietic progenitors as compared to the unfractionated population. All types of myeloid, erythroid and thrombocytic progenitor cells developed from the double-positive αIIb+ $CD45^+$ cell population.

Presence of αIIb on early progenitors thus appears as a useful marker to sort out avian HPC and to trace their early appearance during embryogenesis.

We next demonstrated the αIIb expression on myeloid and erythroid progenitors from bone marrow (BM). Indeed in addition to thrombocytic progenitors, αIIb positive E14 or adult BM cells also contained myeloid and erythroid progenitors. As c-Kit is expressed on mammalian HPC, we double stained with anti-αIIb and -c-Kit Mabs and showed that the anti-αIIb Mab selects multilineage progenitors within the c-$Kit^+$ BM population.

*Lymphoid potential of BM cells*

Avian pro-T cells are contained within the c-$Kit^+$ embryonic BM cell population (Vainio *et al.,* 1996). In collaboration with Drs B. Imhof and C. Ody, we searched whether $\alpha IIb^+$c-$Kit^+$ BM cell populations contained T cell progenitors by using the experimental procedure illustrated in Figure 2A. We demonstrated, for the first time, that $\alpha IIb^+$ cells from embryonic and adult BM could differentiate into T lymphocytes (Table 2).

All these results made it clear that the αIIb integrin can no longer be considered as an exquisite marker for the thrombocytic lineage. Instead, αIIb integrin is expressed by different HPC, including lymphoid progenitors.

***Mouse***

*Erythro-myeloid progenitor activity in $\alpha IIb^+$ cells from mouse FL and BM*

We showed that $\alpha IIb^+$c-$Kit^+$ cells from E13 FL, which represented 1.2%±0.6% of nucleated cells, contained myeloid progenitors i.e. CFU-G, -M, -GM, erythroid progenitors i.e., BFU-E and mixed progenitors i.e., CFU-Mix.

In BM, $\alpha IIb^+$c-$Kit^+$ cells, whose percentage was similar to that in FL i.e., 0.9%±0.3%, also contained erythroid, myeloid and mixed progenitors.

*T-lymphoid progenitor activity in $\alpha IIb^+$ cells from FL and BM*

In order to determine whether T cell progenitors expressed αIIb and c-Kit, a novel two-step method based on *in vitro* and *in vivo* assays was performed as shown in Figure 2B.

To enrich in precursor cells, the BM cell suspension was first depleted of the more mature stages of different lineages with specific antibodies ($Lin^-$). The $Lin^-$c-$Kit^+\alpha IIb^+$ sorted cells were co-cultured with E10 thymic rudiments for 17 days. Donor type Thy-$1.1^+$ cells developed which did not express CD3, CD4 and CD8, the majority was $CD25^+$ (approximately 80%) and a subset expressed CD44 (approximately 17%). This indicates that the

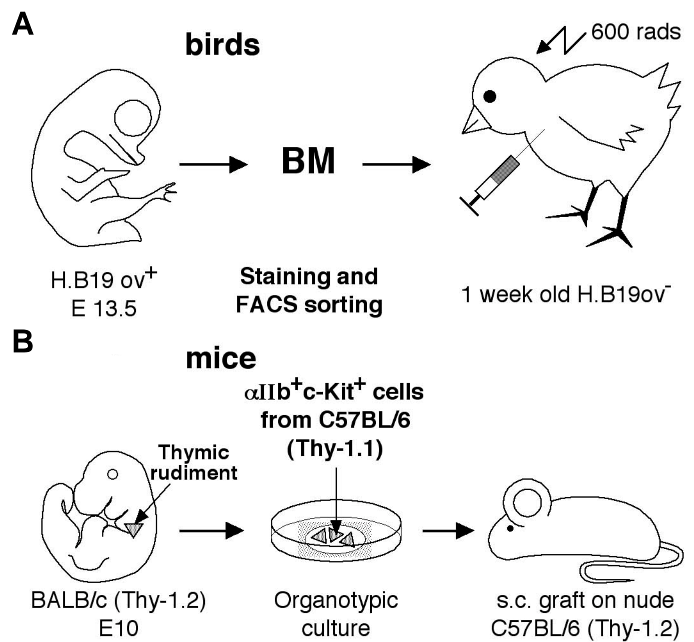


**Fig. 2. Schematic representation of methods used for T cell progenitors. (A)** *Avian assay. In vivo T cell progenitor assay by intrathymic injection was done as described (Vainio* et al., *1996).* **(B)** *Murine assay. BALB/c (Thy-1.2) E10 thymic epithelial rudiment, devoid of HC were dissected out and placed in organ cultures on a filter at the surface of a steel grid. Sorted C57BL/6 (Thy-1.1) $\alpha IIb^+$c-$Kit^+$ cells were deposited on top of thymic anlage. Nine to 17 days later, the thymic cultures were grafted under the skin of 2-week-old C57BL/6 nude Thy-1.2 as described (Salaün* et al., *1990). Three to 4 weeks later, chimerism in the grafted thymus and peripheral organs was analyzed.*

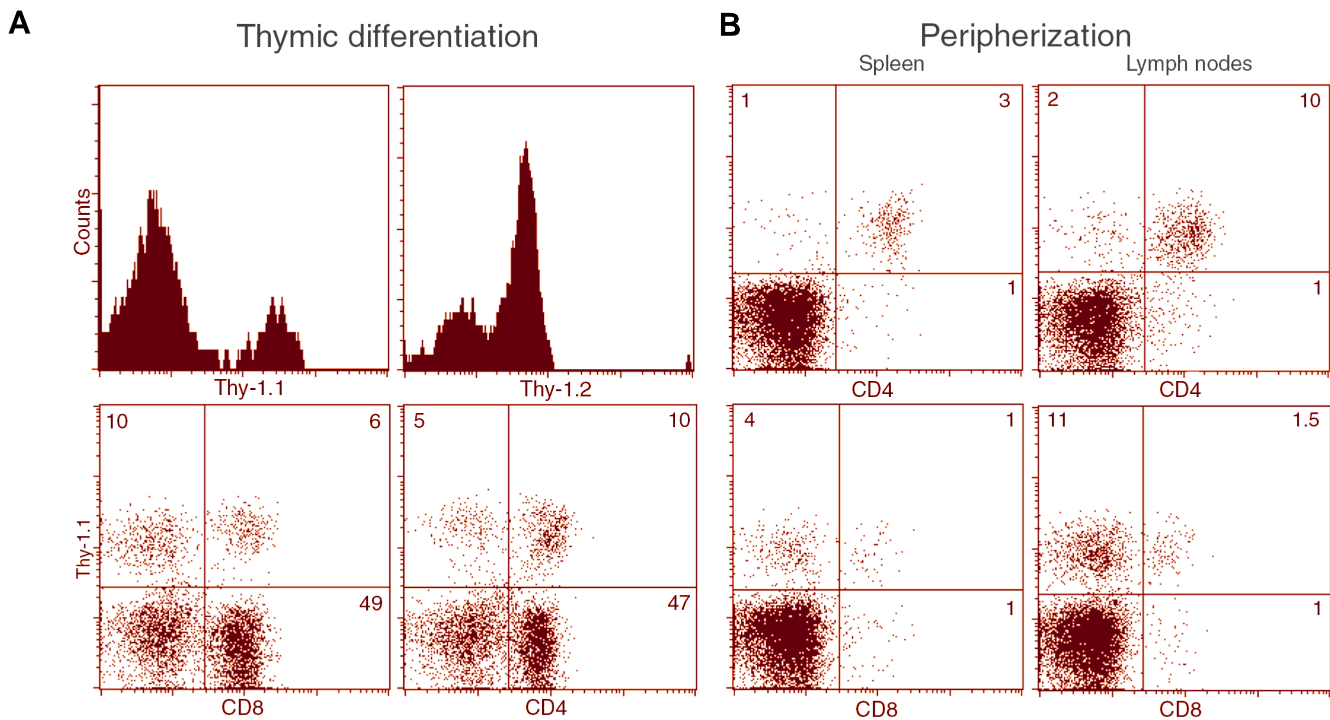


**Fig. 3. T cell differentiation from c-Kit+αIIb+ E13 FL cells. (A)** *FACS analysis of developing T cells in the grafted thymus of one representative chimera. 6x10$^3$ c-Kit+αIIb+ cells from C57BL/6 B.A (Thy-1.1) mice were organ-cultured on E10 BALB/c (Thy-1.2) thymic rudiments during 9 days, which were s.c. grafted onto C57BL6 (Thy-1.2) nude mice for 3 weeks. Donor Thy-1.1+ cells and recipient Thy-1.2+ cells (upper part). Double staining of Thy-1.1+ cells for either CD8 or CD4 expression (lower part).* **(B)** *FACS analysis of developing T cells in the spleen and the lymph nodes of the same chimera. Cells were double stained for donor Thy-1.1 and either CD4 or CD8.*

most immature thymocytes CD3$^-$CD4$^-$CD8$^-$CD25$^+$ and/or CD44$^+$ (Rodewald and Fehling, 1998) differentiated from Lin-c-Kit$^+$αIIb$^+$ BM cells in this early thymic microenvironment.

For further differentiation and maturation of the T cell lineage, a second step was performed *in vivo*. 19 days more *in vivo*, donor type Thy-1.1$^+$ cells developed and acquired CD8 (25%) and CD4 (38%). Therefore, in these experimental conditions, the c-Kit$^+$αIIb$^+$ cell fraction was able to colonize the thymic epithelium and give rise to CD4$^+$ and CD8$^+$ thymocytes.

The ability of c-Kit$^+$αIIb$^+$ cells from E13 FL to differentiate into T lymphocytes was also studied as shown in Figure 3. Colonized thymic lobes were analyzed after 9 days *in vitro* and 27 days *in vivo*. Approximately 15% Thy-1.1$^+$ cells were of donor type and acquired CD8 (5%) and CD4 (10%). Thy-1.2$^+$ derived from host progenitors also developed and represented the Thy-1.1$^-$CD8$^+$ and Thy-1.1$^-$CD4$^+$ thymocytes (49% and 47% respectively) (Fig. 3A).

We then investigated whether donor type thymocytes had migrated to the periphery. Donor T lymphocytes migrated in the spleen and in the lymph nodes where 4% and 12% Thy-1.1$^+$ cells were found, respectively (Fig. 3B). The majority of donor type cells was CD4$^+$ and these cells were single positive lymphocytes. Therefore after 3 weeks, donor-derived T cells had matured sufficiently to be exported from the grafted thymus.

Altogether, these results demonstrate that c-Kit$^+$ progenitor cells expressing αIIb from E13 FL have the capacity first to seed and populate an uncolonized E10 epithelial thymic rudiment *in vitro*, secondly to expand and develop *in vivo* into athymic nude host, along the T lineage pathway, acquiring Thy-1.1, CD4 and CD8 intrathymically. These T lymphocytes differentiated from the αIIb progenitors are able to migrate to the periphery.

*Potentialities of c-Kit $^+$αIIb $^+$ adult BM cells transplanted into irradiated mice*

Moreover, it was important to study the HSC activity of αIIb$^+$ HPC. By definition HSC activity is demonstrated by the ability of sorted cells to provide long-term multilineage hemopoietic reconstitution when transplanted into an adult animal and self-renew.

We thus tested the ability of c-Kit$^+$αIIb$^+$ adult BM cells to contribute to long-term repopulation of irradiated host and to self-renew in a competitive reconstitution assay. As shown in figure 4,

TABLE 2

**PERCENTAGE OF CHIMERISM AFTER INTRATHYMIC INJECTION OF CHICK BM SORTED CELLS**

| BM Cell Type | Sorted Cells | Cells Injected /Thymic Lobe | % of Chimerism |
|---|---|---|---|
| | c- Kit$^+$ αIIbβ3$^-$ | 1000 | 3.7 ± 0.8 |
| E14 | c- Kit$^+$ αIIbβ3$^+$ | 1000 | 20.9 ± 4.3 |
| | c- Kit$^+$ αIIbβ3$^+$ | 100 | 5.8 ± 2.0 |
| | c- Kit$^+$ αIIbβ3$^-$ | 1000 | 0.1 |
| Adult | c- Kit$^+$ αIIbβ3$^{+lo}$ | 1000 | 10.0 ± 3.9 |

Sorted cells from H.B19 ov$^+$ chickens were injected into the thymus of 8-day-old irradiated congenic H.B19 ov$^-$ chickens. The chimerism of the recipient's thymus was determined 2 weeks later by FACS with Mab which recognizes the ov$^+$ antigen (see Fig. 2A).

1000 c-Kit$^+\alpha$IIb$^+$ cells resulted in engraftment of 6 out of 10 recipients after 8 and 16 weeks and 4 mice after 32 weeks, defining a positive recipient as a recipient with more than 1% donor type Ly-5.1. Eight out of 10 mice transplanted with 7500 c-Kit$^+\alpha$IIb$^+$ cells had Ly-5.1 PBL derived from this population after 6 weeks. At 16 and 32 weeks, 7 mice remained positive. The percentage of donor Ly-5.1 was however very low and the level of donor contribution decreased with time, except in one case. Thus c-Kit$^+\alpha$IIb$^+$ cells appear to have only short–term hemopoietic activities.

All mice that showed engraftment from 1000 or 7500 transplanted BM c-Kit$^+\alpha$IIb$^+$ cells contained donor type cells of multiple lineages, consisting of both myeloid and lymphoid cells in spleen and blood. This confirms that myeloid and T lymphoid progenitors express c-Kit and $\alpha$IIb and demonstrate that c-Kit$^+\alpha$IIb$^+$ cells have also the ability of differentiate into the B lymphoid lineage. However these cells were deprived of long-term reconstitution ability.

## Discussion

The $\alpha$IIb$\beta$3 integrin chain has been considered as the hallmark of the thrombocytic/megakaryocytic lineage. We have demonstrated (Ody *et al.*, 1999; Corbel and Salaün, 2002) that it is also expressed by progenitors of the myeloid, erythroid and lymphoid lineages in the embryonic and adult BM but also in the FL. In the embryo it has come to the fore as a particularity sharp marker for intra-aortic clusters, since labelling is restricted to the hemopoietic cells in these clusters. Moreover, we showed that in early chick embryos, myeloid and erythroid intra-aortic progenitors were highly enriched in the $\alpha$IIb$^+$ cell population. Upon double staining with anti-$\alpha$IIb and -CD45 Mabs, three populations were sorted. Progenitors giving rise to myeloid and erythroid colonies were only in the population expressing both $\alpha$IIb and CD45.

Parallel stainings for $\alpha$IIb and $\alpha$V reveal perfectly complementary patterns in hemopoietic clusters and endothelial cells of the aorta (Corbel, 2002). It should be noticed that this pattern is different from that of VEGFR2, an endothelial marker which disappears from the hemopoietic area of the chicken aorta (Jaffredo *et al.*, 1998).

In the chicken embryo, 11C3 Mab (Lacoste-Eleaume *et al.*, 1994) is the first antibody against $\alpha$IIb$\beta$3 capable of uniquely detecting hemopoietic aortic clusters since CD45 also stains abundant scattered cells in the embryo, which probably belong to the monocyte lineage (Cuadros *et al.*, 1992). It is also the first antibody that discriminates the intra-aortic clusters from the underlying endothelial cells.

In the mouse, $\alpha$IIb is also a useful marker for the intra-arterial clusters. Histological detection of these hemopoietic clusters was investigated previously. However, the investigations analyzed the expression of molecules which are not restricted to HC but shared with other cell lineages, either the endothelial lineage (CD31, CD34) or with more differentiated hematopoietic cells (CD45) (Garcia-Porrero *et al.*, 1998; Wood *et al.*, 1997; North *et al.*, 1999; de Bruijn *et al.*, 2000; Manaia *et al.*, 2000; Tavian *et al.*, 1996).

Furthermore, in both chicken and mouse models, cell sorting on the basis of $\alpha$IIb establish the antibody against $\alpha$IIb as a sensitive tool to sort out progenitors with multilineage potential.

We provided the first evidence in birds and in mice that $\alpha$IIb is expressed by BM progenitors belonging not only to the myeloid and erythroid lineages but also to the T lymphoid lineage. $\alpha$IIb expressing c-Kit$^+$ cells from mouse FL were also able to differentiate into different hemopoietic cell types including lymphocytes. Although BM multilineage progenitors express the $\alpha$IIb integrin, they do not behave as long-term, self-renewing HSC but rather as short-term HSC. The ability of intra-embryonic $\alpha$IIb expressing cells to long-term reconstitute lethally irradiated mice remains to be determined.

The potential of $\alpha$IIb expressing HPC might depend on the developmental stage i.e. early embryo, fetus or adult. Indeed, differences occurred between $\alpha$IIb$^+$CD34$^+$ cells from human cord blood and BM. Multipotent HPC were found in the double positive population from neonates while HPC from BM were more restricted to erythroid and megakaryocytic lineages (Debili *et al.,* 2001).

In E10 mouse embryos, HC inside the blood islands from yolk sac expressed $\alpha$IIb (Corbel and Salaün, 2002). This was also observed by groups who analyzed the hemopoietic potentialities of $\alpha$IIb$^+$ yolk sac and embryoid bodies cells by means of clonogenic assays (Mitjavila-Garcia *et al.*, 2002; Mikkola *et al.*, 2003; Ferkowicz *et al.,* 2003). Some investigators now consider that $\alpha$IIb is the earliest marker for hemopoietic commitment, both during embryonic development and in the hierarchy of lineage differentiation.

In mice, we did not perform progenitor potentiality analysis but co-localization of $\alpha$IIb and CD45 was studied at the level of the intra-arterial clusters. Most of them co-expressed the two molecules but the latter was more weekly expressed. This suggested that $\alpha$IIb is either expressed before (or at the same time) as CD45. It is indeed striking that by using the Cre-LoxP strategy to trace the expression of $\alpha$IIb (gpIIb) (Emambokus and Frampton, 2003), the early pattern of $\alpha$IIb expression was identical to what we demonstrated.

A functional role of $\alpha$IIb on hemopoietic progenitors might be to mediate adherence to extracellular matrix molecules such as fibronectin or vitronectin. Recently, conditional ablation of the $\alpha$IIb gene was performed in order to assess its function on HPC. A regulatory role was assigned to $\alpha$IIb since more progenitors were produced when $\alpha$IIb was absent, while VLA-4 and VLA-5 fibronectin receptors were expressed normally. However the adhesive function of these fibronectin receptors was lost in the mutant mice (Emambokus and Frampton, 2003).

Altogether, our data indicate a role for this integrin at early stages in the ontogeny of the hemopoietic system both in chicken and mice. The question of the role of $\alpha$IIb signaling in determining the number and lineage distribution of HPC represents an interesting area of research. In the immediate future, $\alpha$IIb appears as a novel marker for HPC, which is especially useful for investigators working on the early ontogeny of the hemopoietic system.

*Acknowledgments*

*We are grateful to A. Lehmann and P. Belo for technical assistance; S. Gournet and F. Beaujean for the illustrations; M. Scaglia for her help with manuscripts. C Corbel thanks Drs. B. Imhof and C. Ody (CMU, Geneva, Switzerland) for the fruitful collaboration to the study in the avian model. We are indebted to Dr. F. Dieterlen for critical and helpful discussions throughout these studies.*

*This work was supported by the Centre National de la Recherche Scientifique and the Institut National de la Santé et de la Recherche Médicale.*

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