Molecular dissection of Cdc6 and the miR-148 family : two stories with common themes

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Chapter 5

Differential expression of miR-148 in hematopoietic thymic subsets and its potential function

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Differential expression of miR-148 in hematopoietic thymic subsets and its potential function

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MicroRNAs (miRNAs) have been implicated in several cellular processes, such as cell proliferation, differentiation and apoptosis. miR-148 was found expressed in the spleen and enriched in human cell lines of hematopoietic origin. Interestingly, we identified Dnmt3b and MLL (mixed lineage leukaemia) as possible miR-148 targets and they both appear to be involved in T cell development and survival. To study whether miR-148 mediated repression of Dnmt3b and MLL plays a role in human lymphoid development, we determined the expression of miR-148, Dnmt3b and MLL in different lymphoid subsets isolated from human postnatal thymus. Whereas miR-148 is highly expressed in CD34+CD1a- early thymic progenitor cells and remains expressed in all subsets of T cell committed lineages, its expression is significantly decreased in natural killer (NK) cells and plasmacytoid dendritic cells (pDCs). However, we found that Dnmt3b and MLL mRNA levels are reduced in these subsets as well. This suggests that relieve of miR-148 mediated repression of Dnmt3b and MLL is not a key step in pDC and NK development. Instead, miR-148 might play a role in fine-tuning the expression of these targets in lymphoid development. To test whether reduction of miR-148 level is essential for pDC development we exogenously expressed miR-148 in mTPCs and performed in vitro pDC differentiation assays. Interestingly, miR-148 overexpression resulted in a dramatic increase in both percentage and number of pDCs.

Introduction

Currently, several hundreds of miRNAs have been identified, and they have been implicated in many cellular processes such as cell proliferation, apoptosis and differentiation (Carleton et al., 2007). Whereas some miRNAs are broadly expressed, others are expressed in a tissue-specific manner (Landgraf et al., 2007). To gain more insight in the role of miRNAs in development and differentiation, a number of studies examined the expression of miRNAs in cells of the immune system. In a first large-scale attempt to identify miRNAs involved in mammalian hematopoiesis approximately 100 miRNAs were cloned from mouse bone marrow (Chen et al., 2004). Three of these miRNAs showed preferential expression in hematopoietic tissue compared to tissues of other origins. Ectopic expression in a hematopoietic precursor cell of one of these miRNAs, miR-181, resulted in a significant increase in B cell development (Chen et al., 2004). In another study, several miRNAs were shown to be down-regulated following in vitro differentiation from CD34+ bone marrow cells to megakaryocytes (Garzon et al., 2006). Further, miR-155 expression was shown to regulate the germinal center reaction, a specific differentiation processes in the immune response (Thai et al., 2007).

Finally, miR-150, which is specifically expressed in mature lymphocytes and not in their progenitors was shown to control B cell differentiation by regulating its target c-Myb (Xiao et al., 2007). miR-150 overexpression in B-cell progenitors resulted in down-regulation of c-Myb and in a partial block of early B-cell development. Together, these studies indicate that miRNAs can play an important role in differentiation of hematopoietic cells.

Human miR-148a (which we will refer to as miR-148) was originally cloned from the mouse spleen. This expression appeared to be specific, since no clones were derived from heart, liver, small intestine, colon or brain tissue (Lagos-Quintana et al., 2002). In humans miR-148 was also found expressed in the spleen and enriched in human cell lines of hematopoietic origin (Landgraf et al., 2007). This is in accordance with our finding that miR-148a is expressed to the highest extent in Jurkat T cells compared to other cancer cell lines (Chapter 4). Interestingly, we identified a number of miR-148 targets that might play a role in T cell survival and development, such as Dnmt3b (Chapter 4). Dnmt3b mutations in humans were linked to the Immunodeficiency, Centromeric region instability and Facial anomalies (ICF) syndrome (Xu et al., 1999). ICF patients are characterized by low serum immunoglobulin levels and low numbers of peripheral blood B and T cells (Ehrlich, 2003). A mouse model of the ICF syndrome implied that the low number of T cells are the result of T cell death, thereby suggesting that Dnmt3b expression might play a role in T cell survival (Ueda et al., 2006). Furthermore, gene expression profiling by micro-array analysis revealed that Dnmt3b is differentially expressed in consecutive stages of T cell development. Whereas Dnmt3b was highly expressed in early thymic progenitor cells, its expression gradually decreased at more mature stages of development (Dik et al., 2005). Another possible miR-148 target mRNA is MLL (mixed lineage leukaemia) as will be shown here. MLL was identified as a common breakpoint in human leukaemia's and subsequently was shown to be involved in myeloid and lymphoid cancers through its fusion with many genes of other chromosomes (Daser and Rabbitts, 2005). Although less is known about its role in normal hematopoiesis, MLL appears to be essential, since MLL heterozygous mice displayed hematopoietic abnormalities (Yu et al., 1995). Furthermore, MLL is a major activator of class I homeobox (HOX) gene expression, by interacting directly with HOX promoter regions (Milne et al., 2002; Nakamura et al., 2002). For HOX genes also several lines of evidence exist for their involvement in hematopoiesis (Daser and Rabbitts, 2005).

Several cell subsets have been characterized in human thymic development (Figure 1). CD34+CD1a- cells are early multipotent thymic progenitor cells (mTPCs), which have the capacity to give rise to T cells, NK (natural killer) cells and pDCs (plasmacytoid dendritic cells). Upregulation of CD1a resulting in CD34+CD1a+ cells, is associated with T cell commitment, since these cells

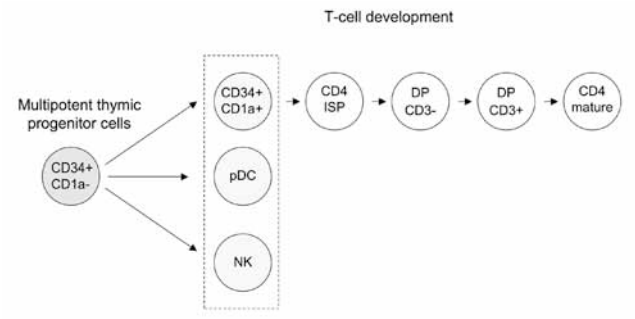

Figure 1. Schematic representation of human thymocyte differentiation.

have little capacity to develop into NK cells and completely lost their pDC developmental potential (Spits et al., 2000). CD34+CD1a+ cells develop into CD4 immature single positive (ISP) cells, CD3- double positive (DP) cells that are positive for both the CD4 and CD8 receptor, CD3+ DP cells and finally they develop into CD4 mature single positive T cells (Blom and Spits, 2006).
We were intrigued by the overlap of miR-148 expression in hematopoietic cells and the possible functions of its targets Dnmt3b and MLL in T cell development and survival. In this study we aimed to analyse the potential role of miR-148 and its targets in more detail in human thymic development.

Results and discussion

miR-148 and Dnmt3b

To study the role of miR-148 and Dnmt3b in early hematopoiesis, cells in different stages of thymic development were isolated from post natal human thymus tissue by flow cytometric sorting. The expression level of several markers was used to isolate cells from different developmental stages as depicted in Figure 1. In addition to these T cell committed cells, pDC (BDCA2+CD123hi) and NK cells (CD56+CD3-) were isolated, which both can arise from mTPCs (Figure 1). We isolated RNA from sorted cell populations, prepared cDNA and performed quantitative Real-Time (RT) PCR analysis for miR-148. We found that mTPCs contain high miR-148 levels (Figure 2A and data not shown). Moreover, the CD34+CD1a+ T cell committed cells showed an increase of miR-148 expression of 2,5 times. However, apart from a slight increase observed in DP CD3- cells compared to mTPCs, we did not observe a significant change of miR-148 expression in other analysed T cell subsets.
We previously showed that miR-148 regulates Dnmt3b1 expression through interaction with its coding region (Chapter 4). Therefore, the same RNA was subjected to quantitative RT-PCR analysis for Dnmt3b1 expression (Figure 2B). Also Dnmt3b1 was highly expressed in mTPCs, however, its expression gradually

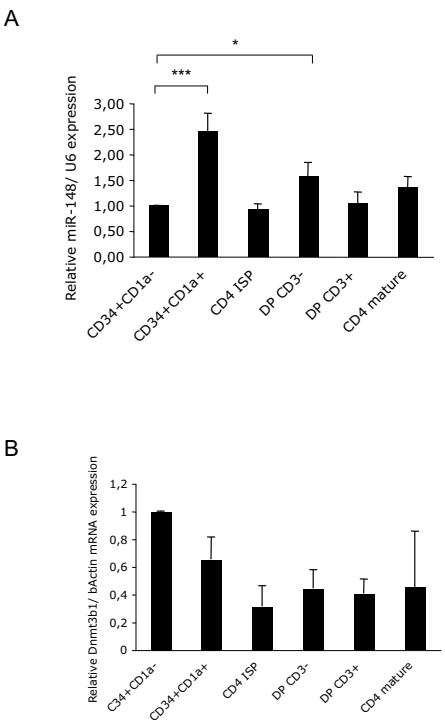

Figure 2. Relative miR-148 and Dnmt3b expression in thymic T cell subsets. A Freshly isolated thymic subsets were analyzed by quantitative RT-PCR for the presence of miR-148 (n=3). Values were calculated relative to CD34+CD1a- cells. Error bars represent standard error of the mean. **B** As descibed in A but analyzed for Dnmt3b1 mRNA expression (n=2). Error bars represent standard deviation. * P<0.05 *** P<0.001

decreased in developing T lymphocytes. This result is in line with previously published micro-array data, were similar T cell subsets from the human thymus were analysed for mRNA expression (Dik et al., 2005). Compared to CD34+CD1a- cells Dnmt3b1 mRNA level decreases in the CD34+CD1a+ cells whereas miR-148 level goes up. Yet, in other early T cell subsets no clear correlation was observed.
Next, we analysed miR-148 expression in pDC and NK cells, which arise from the same mTPC (Figure 1). Interestingly, we detected a seven fold and a five fold reduction in

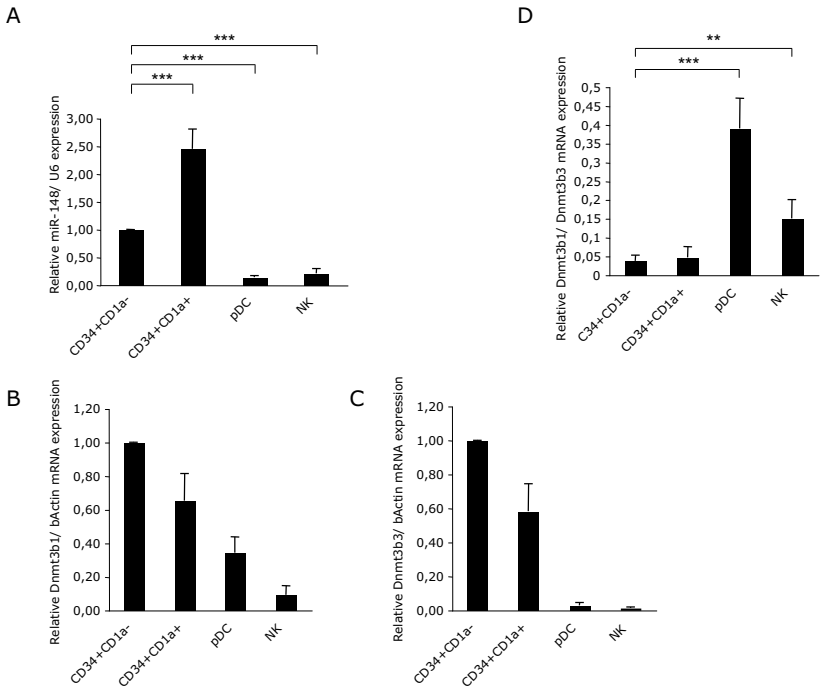

Figure 3. Relative miR-148, Dnmt3b1 and Dnmt3b3 expression in early thymic subsets. A Freshy isolated thymic subsets were analyzed by quantitative RT-PCR for the presence of miR-148 (n=3). Values were calculated relative to CD34+CD1a- cells. Error bars represent standard error of the mean. **B** and **C** As described in A but analyzed for Dnmt3b1 and Dnmt3b3 (n=2). D Relative Dnmt3b1/Dnmt3b3 ratio calculated from Dnmt3b1 and Dnmt3b3 values relative to CD34+CD1a- Dnmt3b1. ** P<0.01 *** P<0.001

miR-148 expression in pDC and NK cells, respectively (Figure 3A). Since miR-148 expression increased 2,5 times in T cell committed CD34+CD1a+ cells (Figure 2A), this result shows differential expression of miR-148 in different early thymic subsets, which all arise from the same pool of progenitor cells.

In addition, we examined Dnmt3b1 expression in the pDC and NK cells and found a reduction of its expression in both pDC and NK cells (Figure 3B). This suggests that also in pDC and NK cells Dnmt3b1 mRNA level is regulated by other means than miR-148 expression.

We previously described that miR-148 targets a site in the Dnmt3b1 coding region that is absent from the Dnmt3b3 splice variant mRNA (Chapter 4). Dnmt3b3 mRNA expression appears to be down-regulated in pDC and NK cells as well (Figure 3C). However, whereas the ratio of Dnmt3b1 and Dnmt3b3 was similar in CD34+CD1a- cells and CD34+CD1a+ cells, the relative abundance of Dnmt3b1 to Dnmt3b3 increased dramatically in pDC and NK cells (Figure 3D). This alteration in balance of Dnmt3b splice variants, could reflect the significant reduced miR-148 expression in pDC and NK cells. Yet, a role of changed splicing activity in the different hematopoietic subsets can not be excluded.

Currently it is unknown whether Dnmt3b splice variants play a role in Dnmt3b function. The role of the highly expressed and miR-148 resistant Dnmt3b3 splice variant in DNA methylation is particularly interesting. Some discrepancy has been reported on whether

human Dnmt3b3 is catalytically active or inactive depending on the substrate chosen (Chen et al., 2005; Soejima et al., 2003). This could reflect a change in target preference compared to Dnmt3b1 since Dnmt3b3 lacks a motif that could be involved in target recognition (Kumar et al., 1994). Further, it is interesting to note that overexpression of the Dnmt3b4 splice variant has been associated with dominant negative regulation, since hypomethylation of pericentromeric satellite regions was observed (Saito et al., 2002). Alternatively, this lack of satellite methylation could reflect a change in target preference. Since Dnmt3b3 and Dnmt3b4 lack the same target recognition motif, it would be interesting to determine whether Dnmt3b3 like Dnmt3b4 regulates target preference or has dominant negative activity. If so, it would be interesting to study the altered balance of Dnmt3b1 and Dnmt3b3 in more detail in pDC and NK development.

miR-148 and MLL

MLL was predicted by Targetscan 3.0 to be a likely miR-148 target. To test this experimentally, the MLL 3'UTR was cloned behind Luciferase in the pGL3 vector. Co-transfection of this vector with miR-148 resulted in a reduction of Luciferase activity of almost 50% compared to the empty pGL3 vector (Figure 4A). According to the prediction, two miR-148 target sites exist in the MLL 3'UTR. Deletion of 140 basepairs surrounding these sites of the 3 kb total MLL 3'UTR, completely abolished miR-148 dependent MLL regulation (Figure 4A). This suggests that miR-148 is indeed able to directly regulate MLL mRNA expression by interacting with at least one of these sites in its 3'UTR.

To test whether miR-148 could induce degradation of endogenous MLL mRNA, miR-148 was transfected into HeLa cells. Three days after transfection, RNA from these cells was isolated and cDNA was prepared

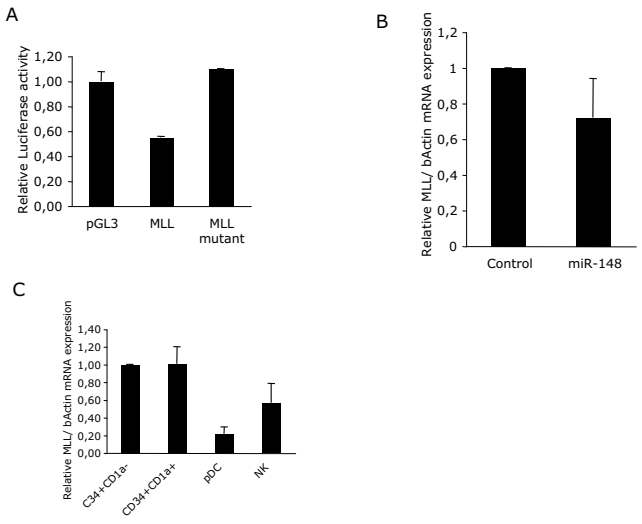

Figure 4. miR-148 mediated regulation of MLL and MLL expression in early thymic subsets. A miR-148 represses pGL3-Luc expression with the wild-type MLL 3'UTR (MLL) but not a mutant with mutated miR-148 target sites (MLL mutant). Expression was calculated relative to a co-transfection with a control miRNA. A representative experiment is shown. Error bars represent standard deviation. **B** Representative quantitative RT-PCR of endogenous MLL expression in HeLa cells transfected with a control miRNA or with miR-148. Error bars represent standard deviation. **C** Relative MLL expression was calculated in freshly isolated early thymic subsets (n=1). Error bars represent standard deviation.

for quantitative RT-PCR. The expression of MLL was 30% reduced in the presence of miR-148 suggesting that at least part of the miR-148 regulation is mediated through decreased mRNA stability (Figure 4B).
Next, we tested by quantitative RT-PCR whether MLL mRNA expression was affected in pDC or NK cells. Although miR-148 was considerably decreased in pDC and NK cells compared to mTPCs, we observed both in pDC and NK cells a down-regulation of MLL expression (Figure 4C). No change in MLL mRNA level was observed in CD34+CD1a+ cells compared to mTPCs.
The down-regulation of MLL expression in pDC and NK cells was less pronounced than the Dnmt3b down-regulation in these cells (Figure 3B). Nevertheless, it is a similar trend for both miR-148 targets. This suggests that miR-148 is highly expressed in cells with high Dnmt3b and MLL mRNA levels and low expressed in cells with low Dnmt3b and MLL mRNA levels. Notably, also human stem cell like testicular germ cells express high levels of miR-148 and these cells express high levels of Dnmt3b (Chapter 4 and data not shown).
Different kind of miRNA and mRNA interactions have been described so far (Plasterk, 2006). Some interactions might result in cell-fate switches during development and these interactions are under positive evolutionary pressure. Although the targeting of Dnmt3b and MLL by miR-148 appears to be evolutionary conserved (chapter 4 and Targetscan 3.0), these interactions are most likely not part of a switch in pDC and NK cell development from mTPCs.
Alternatively, it has been proposed that miRNAs and their targets can be induced by the same pathway. It has been suggested that this mechanism controls the fine-tuning of target gene expression (Hornstein and Shomron, 2006). An example is c-Myc that simultaneously induces the expression of E2F1 and of miR-17-5p and miR-20a that target E2F1 (O'Donnell et al., 2005). Likewise, miR-148, Dnmt3b and MLL might be co-regulated and miR-148 might play a role in fine-tuning the expression of these targets in early thymic subsets.

Exogenous miR-148 expression results in increased pDC cell numbers

Since miR-148 is down-regulated in pDC cells compared to CD34+CD1a- mTPCs, we were interested to determine the effect of miR-148 overexpression on pDC development. To study this we made use of an in vitro pDC differentiation assay (Schotte et al., 2003; Spits et al., 2000). We transduced CD34+CD1a- mTPCs derived from human post natal thymus with miR-148. The mixture of transduced and non-transduced cells were allowed to differentiate into pDC by coculturing with the murine bone marrow stromal cell line OP9 in the presence of the cytokines IL-7 and Flt3L. miR-147 and a construct containing a non-functional part of human Telomerase RNA (hTR) were used as controls and all three vectors contained a YFP marker. Transduced mTPCS were counted and analysed for YFP expression on day 0 and day 11. A slight increase in YFP positive cells was observed at day 11 in both control transduced cells. However, in the miR-148 transduced cells a dramatic increase in the percentage of YFP positive cells of almost 8 fold was observed (data not shown). A similar increase was observed for absolute cell numbers (data not shown). Staining for the pDC markers CD123high BDCA2+ showed increased percentages of pDCs (Figure 5A). Interestingly, calculating the absolute cell number of pDCs in the transduced populations revealed that forced miR-148 expression gave rise to 15-fold higher numbers of pDCs compared to the hTR and miR-147 expressing cells (Figure 5B).
miR-148 transduced YFP positive cells that were not positive for the pDC markers showed only a slight increase of four times in relative expansion (data not shown), indicating that the observed effect was most pronounced in pDC cells. Together, these results show that overexpression of miR-148 strongly increases the number of pDCs that are derived from CD34+CD1a- mTPCs.

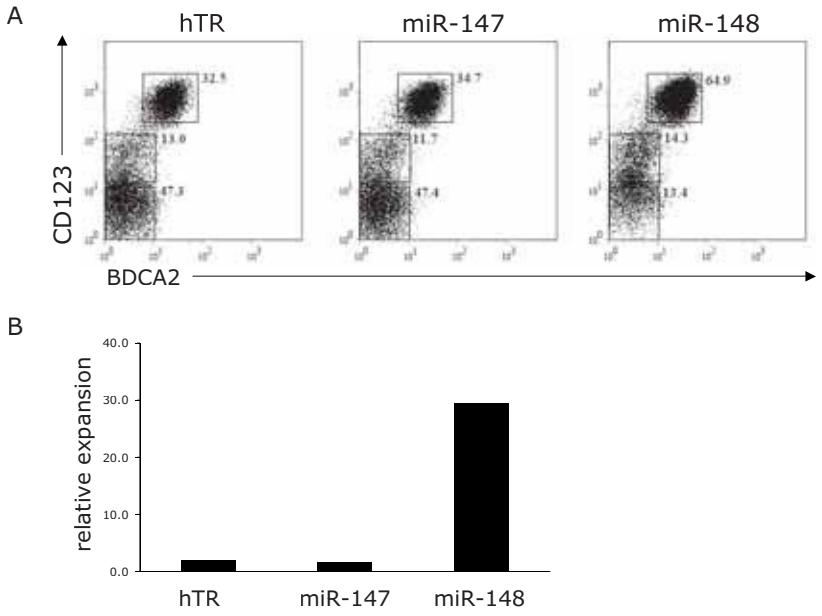

Figure 5. miR-148 overexpression results in an increased number of pDCs. A hTR, miR-147 and miR-148 transduced CD34+CD1a- cells were cocultered with OP9 cells in the presence of IL-7 and Flt3L and analysed after 11 days for the presence of pDCs (CD123highBDCA2+) **B** Relative expansion of pDC cells. Fold expansion in absolute cell numbers of the pD subset in the transduced population was calculated on basis of total numbers of cells harvested from the cultures after 11 days, percentages of transduced cells, and percentages of each population corrected for the number of input cells

Whether this effect is due to enhanced differentiation, proliferation or survival of pDCs remains to be determined. Furthermore, these results imply that miR-148 is down-regulated in pDCs to limit the number of pDCs in the human thymus. The main function of pDCs in the immune response is their capacity to produce high levels of type I interferons, which directly inhibits viral replication and triggers T cell mediated responses (Blom et al., 2002; Kadowaki et al., 2000). However, in the thymus pDC activation has recently been shown to impair thymic T cell development (Schmidlin et al., 2006). Therefore, miR-148 downregulation in pDCs could ensure proper thymic T cell development by keeping the numbers of pDCs low. In case miR-148 expression leads to increased survival and/or proliferation of pDCs it would be interesting to test pDC leukemic cells for expression of miR-148 (Chaperot et al., 2001). What remains to be determined is via which targets miR-148 exerts this function in pDCs and whether reduced Dnmt3b1 or MLL are involved. Furthermore, considering the similar expression profile of miR-148 and its targets in pDCs and NK cells it would be interesting to see whether miR-148 has a similar stimulatory effect on NK cell development as it has on pDCs.

Material and Methods

Constructs

All miRNAs were expressed from a retroviral miR-Vec vector expressing Blastacidin or YFP as described before (Voorhoeve et al., 2006). The MLL 3'UTR was cloned downstream the luciferase gene in AatII and AgeI sites generated in the pGL3 constructs. The following primers were used to clone MLL from genomic DNA:

MLL 3'UTR for
GCGCGACGTCAGCTGCTCTTCTCCCCCAGTGT
MLL 3'UTR rev
GATCACCGGTGGGGGATTCTTGGGAATGACCCATC
MLL mutant was generated by using endogenous SpeI and EcoRI sites. Digestion and religation deleted 140 nucleotides.

Cell culture and transfection

Hela cells were grown in Dulbecco Modified Eagle supplemented with 10% fetal bovine serum. Hela cells were transfected with Fugene (Roche) using manufacturer's protocol. For luciferase assays cells were cultured in 24-well plates and transfected one day after seeding with 5 ng pcDNA3-Renilla, 50 ng of the indicated pGL3 Luciferase construct and 200 ng of miR-Vec-148a or a control miRNA construct. Luciferase activity was measured 48 hours after transfection using the Dual-Luciferase reporter assay system (Promega).

RNA isolation and quantitative RT-PCR

RNA was isolated from cells using Trizol (Invitrogen) and 20 µg of glycogen was used for precipitation. Standard cDNA was prepared from 1.5 µg RNA of Hela cells, 0.5 or 1 µg RNA of T cell subsets of RNA using the standard protocol with 2 ul of random hexamer primers (Superscript III first-strand synthesis system for RT-PCR, Invitrogen). DTT was not added to the cDNA reaction to prevent interference with SYBRgreen. Real-time RT-PCR was performed with a standard 2-step amplification protocol of a MiniOpticon System (Bio-Rad) apparatus using a SYBRgreen PCR master mix (Applied Biosystem) and specific primers.
Dnmt3b1 forward GCCGTTCTTCTGGATGTTTGAG
Dnmt3b1 reverse ATCCTATTGTATTCCAAGCAGTCC
Dnmt3b3 forward ATCTCACGGTTCCTGGAGTG
Dnmt3b3 reverse AAGCCAAAGATCCTGTTCATCC
MLL forward CATTGATGCAGGTGAGATGG
MLL reverse GTGATTGATGAAGCGTGCAG
Beta Actin forward CCTGGCACCCAGCACAAT
Beta Actin reverse GGGCCGGACTCGTCATACT
The mirVana qRT-PCR miRNA detection kit (Ambion) was used to detect miRNA expression by quantitative RT-PCR. Specific miR-148a and U6 RT and PCR primers were used (Ambion) and 25 ng of RNA was used per reaction.

Reagents and monoclonal antibodies

Monoclonal antibodies to CD3, CD4, CD8, CD45RA and CD123, conjugated to FITC, PE, PeCy7, APC, or APCCy7 were purchased from Becton Dickinson (BD, San Jose, CA). CD1a-PE was obtained from Coulter/Immunotech (Luminy, France). FITC or APC conjugated anti-BDCA2 and anti-BDCA4 was obtained from Miltenyi Biotec (Bergisch Gladbach, Germany).

Isolation of postnatal thymic subsets

The use of postnatal thymus tissue was approved by the Medical Ethical Committee of the Academic Medical Center. Thymocytes were obtained from surgical specimens removed from children up to 3 years of age undergoing open heart surgery, with informed consent from patients in accordance with the Declaration of Helsinki. The tissue was disrupted by mechanical means and pressed through a stainless steel mesh to obtain a single-cell suspension, which was left overnight at 4°C. The following day thymocytes were isolated from a Ficoll-Hypaque density gradient (Lymphoprep; Nycomed Pharma, Oslo, Norway). Subsequently, thymic subsets were isolated by MACS separation and subsequent cell sorting using a FACSAria (BD) as described below. Purity of the sorted cells in all experiments was greater than 99%.

CD34+1a- and CD34+1a+ subsets

CD34+ cells were enriched by immunomagnetic cell sorting, using a CD34 cell separation kit (varioMACS, Miltenyi Biotec). The CD34+ thymocytes were stained with antibodies against CD34, CD1a, CD56, and BDCA2. CD56-BDCA2- CD34+CD1a- (CD34+CD1a-) and CD56-BDCA2-CD34+CD1a+ (CD34+CD1a+) populations were sorted to purity.

CD4ISP and CD4SP T cells

CD4 immature single positive (CD4ISP) and mature CD4 single positive (CD4SP) T cells were sorted after CD8+ enrichment using a CD8 separation kit (Miltenyi Biotec) CD56-BDCA2-CD8-CD4+CD1a+ (CD4+CD1a+) and CD56-BDCA2-CD8-CD4+CD1a- (CD4+CD1a-) respectively.

CD3- and CD3+ DP T cells

Without prior MACS selection CD3- and CD3+ double positive T cells were sorted based on CD56-BDCA2-CD4+CD8+CD3- (CD3- DP) and CD56-BDCA2-CD4+CD8+CD3+ (CD3+ DP).

Natural Killer (NK) cells

CD56 positive cells were enriched by using the CD56 separation kit (Miltenyi Biotec) and subsequently sorted for NK cells on basis of CD56+CD3-. IL7R+ NK cells (described by Vosshenrich, Nature Immunology, 2006) were excluded by sorting negative for CD127.

Plasmacytoid Dendritic cells (pDC)

For the isolation of pDCs, BDCA4+ cells were enriched by immunomagnetic cell sorting, using a BDCA4+ cell separation kit (Miltenyi Biotec). The BDCA4+ cell fraction was labeled with anti-CD56, anti-CD123 and anti-CD45RA antibodies and CD56-CD123hiCD45RA+ cells were sorted to purity.

Retroviral transduction and differentiation assays

For transduction experiments CD34+CD1a- postnatal thymocytes were cultured overnight in Yssel medium44 with 5% NHS, 20 ng/mL SCF, and 10 ng/mL IL-7 (Yssel et al., 1984). The following day cells were incubated for 6 to 7 hours with virus supernatant in retronectin-coated plates (30 µg/mL; Takara Biomedicals, Otsu, Shiga, Japan). The development of pDCs was assessed by coculturing CD34+CD1a- (BDACA2-/CD56-/CD3-) thymic progenitor cells with 5 x 104 OP9 cells in MEM(alpha) (Invitrogen) with 20% FCS (Hyclone), 5 ng/mL IL-7, and 5 ng/mL Flt3L. At indicated timepoint the assays was analysed for the presence of pDCs by flow cytometry on a CyAn ADP analyzer (Dako, Fort Collins, Co).

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