

## ORIGINAL ARTICLE

# Expression analysis of surface molecules on human thymic dendritic cells with the 10th HLDA Workshop antibody panel

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Dendritic cells (DC) in the thymus have an important role in the establishment of central tolerance by promoting negative selection of autoreactive T cells and regulatory T-cell differentiation. Whereas human DC have recently been studied in various tissues in more detail, thymic DC subsets are still ill-defined. In the present work, we studied the binding of 71 monoclonal antibodies (mAb) submitted to the HLDA10 workshop to human CD123<sup>+</sup> plasmacytoid DC and the two subsets of conventional DC (cDC, CD141<sup>+</sup> and CD11b<sup>+</sup>) isolated from thymus tissue of infants undergoing corrective heart surgery. Within the panel, we found mAb binding to thymic pDC and both cDC subsets (for example, anti-Clec12A, TIM-3, Clec4A, CCR5, Axl, FLT3), but most of them additionally reacted with other thymic cell types. MAb directed to CD85h (ILT1) and the C-type lectin Clec7A (now CD369) reacted selectively with both cDC subsets, but not with other cells. Only one mAb directed to CD85g (ILT7) stained thymic pDC in a highly specific manner. Clec9A (DNGR1, now CD370) was the only tested HLDA10 antigen exclusively expressed on thymic CD141<sup>+</sup> cDC. The present report summarizes all data obtained.

*Clinical & Translational Immunology* (2015) 4, e47; doi:10.1038/cti.2015.21; published online 30 October 2015

Dendritic cells (DC) in lymphoid and non-lymphoid tissues are professional antigen-presenting cells that are necessary for pathogen recognition and the initiation of primary T-cell immune responses.<sup>1,2</sup> Although DC in the thymus may also have a role in the protection against certain infections, it is the presentation of self-antigens to the developing T-cell pool that makes DC indispensable for the proper establishment of central tolerance. Thymic DC were not only shown to promote negative selection of autoreactive T cells, but may also participate in the induction of regulatory T cells (reviewed in Klein *et al.*<sup>3</sup> and Hadeiba and Butcher<sup>4</sup>). As described for various peripheral organs, human thymic DC are most likely heterogeneous regarding their origin and ontogeny, anatomical localization, capacity to take up and process different forms of antigen, and production of various cytokines. It can be anticipated that these differences influence the responses of the maturing T cells interacting with them. Some of these functional differences of thymic DC populations will be reflected by a differential expression of cell surface molecules.

Similar to other tissues, human thymus contains two major types of DC: plasmacytoid DC (pDC, defined as HLA-DR<sup>+</sup> cells expressing IL-3R $\alpha$  (CD123), but lacking expression of CD11c) and conventional DC (cDC, expressing CD11c and high amounts of HLA-DR).<sup>5,6</sup> Within thymic cDC, differential expression of several cell surface molecules, like DC-LAMP, CD14 or CD11b, pointed to the existence of two different subsets also in the human thymus.<sup>5-7</sup> However, an

unequivocal classification of thymic cDC was lacking because of the absence of highly specific markers.

To be able to consistently classify human thymic cDC, we compared a number of surface molecules used in the past for cDC subset definition in the peripheral blood or tissues, including CD141 (BDCA-3, thrombomodulin) and CD1c (BDCA-1).<sup>8-10</sup> In these comparisons, we also included molecules that specifically describe functionally different cDC subsets in various mouse tissues, namely XCR1 and SIRP $\alpha$ .<sup>11,12</sup> These comparisons revealed that human thymus also contains CD141<sup>+</sup> cDC, which perfectly match the described CD11b<sup>neg</sup> thymic cDC.<sup>7</sup> CD141<sup>+</sup> CD11b<sup>neg</sup> thymic cDC express XCR1, but lack SIRP $\alpha$  (<sup>13</sup> and data not shown) and thus resemble 'cross-presenting' DC in peripheral blood.<sup>14,15</sup> Further, we found that all human thymic CD11b<sup>+</sup> cDC co-express SIRP $\alpha$ , and lack XCR1 expression, and thus phenotypically resemble human peripheral CD1c<sup>+</sup> cDC (<sup>13</sup> and data not shown). Thus, human thymic cDC can consistently be subdivided into two subsets: CD141<sup>+</sup> cDC and SIRP $\alpha$ <sup>+</sup> cDC (which are congruent with CD11b<sup>+</sup> cDC). For practical reason (monoclonal antibody (mAb) availability, greater flexibility for combination with various HLDA10 mAb formats), CD11b (and not SIRP $\alpha$ ) together with CD141 was used for cDC classification in this study. The panel of 71 monoclonal antibodies from the 10th Human Leukocyte Differentiation Antigens workshop (HLDA10) was investigated for binding to freshly isolated human thymic pDC, CD141<sup>+</sup> cDC or CD11b<sup>+</sup> cDC. In this report, the results obtained are summarized.

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Received 16 July 2015; revised 4 September 2015; accepted 6 September 2015

## RESULTS

Because of the low abundance of DC in thymic tissue (approximately 0.2–0.3%), DC were enriched from digested human thymi by Nycodenz-gradient centrifugation to 1–4% pDC (defined as live CD123<sup>+</sup> HLA-DR<sup>+</sup> cells) and 6–10% cDC (defined as live CD123<sup>neg</sup> lineage<sup>neg</sup> HLA-DR<sup>+</sup> CD11c<sup>int/high</sup> cells). The cDC were further subdivided into CD141<sup>+</sup> or CD11b<sup>+</sup> cDC (Figure 1). This enrichment allowed an efficient simultaneous analysis of all primary thymic DC subsets. At the same time, gating on lineage<sup>neg</sup> HLA-DR<sup>+</sup> CD11c<sup>neg</sup> CD141<sup>neg</sup> CD123<sup>neg</sup> cells allowed to analyze other HLA-DR<sup>+</sup> thymic cells, which were not DC (HLA-DR<sup>+</sup> non-DC). Gating on HLA-DR<sup>neg</sup>, but lineage-positive cells encompassed mainly immature T cells, but also CD56<sup>+</sup> NK cells. The results, summarized in Table 1, were highly reproducible, with each of the HLDA10 mAb giving comparable staining profiles on cells of all tested donors. Slight variations between single donors were observed only in the absolute fluorescence intensities or in the percentage of the positive fractions. On the basis of their staining profile, the HLDA10 mAb could be grouped into:

1. mAb binding to all primary thymic DC populations (pDC, CD141<sup>+</sup> cDC and CD11b<sup>+</sup> cDC);
2. mAb binding only to cDC but not to pDC;
3. mAb preferentially binding to a subpopulation of CD11b<sup>+</sup> thymic cDC;
4. mAb exclusively binding to CD141<sup>+</sup> cDC;
5. mAb exclusively binding to pDC;
6. mAb that gave only marginal staining on at least one DC population;
7. mAb that did not bind to any of the primary human thymic DC subsets.

### HLDA10 mAb recognizing antigen expressed on all human thymic DC populations

From all 71 tested HLDA10 mAb, 13 mAb bound their antigen on pDC and on both cDC subsets. Of these, Clec12A (now CD371, 3 test

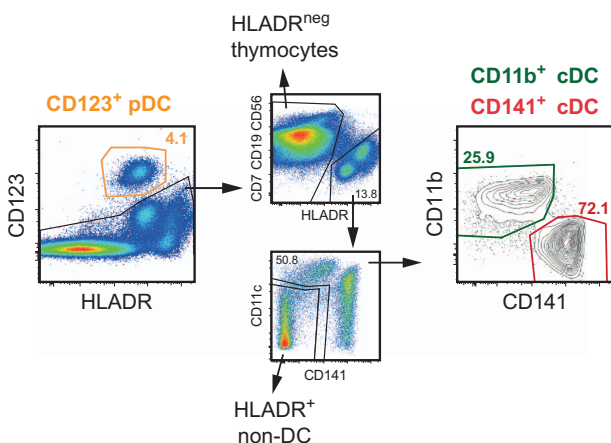
mAb) and TIM-3 (now CD366, 2 test mAb) showed a similar expression level on both cDC subsets and a lower expression on pDC (Figure 2a). The receptor tyrosine kinase FLT3 (CD135), reported to be required for DC development, was found to be one of the few HLDA10-defined molecules clearly expressed at higher levels on thymic CD141<sup>+</sup> cDC, as compared with CD11b<sup>+</sup> cDC (Figure 2b). In contrast, mAb recognizing either Clec4A (DCIR, now CD367), the interleukin-1 receptor accessory protein IL-1RacP (IL-1R3), the myeloid-associated immunoglobulin-like receptor II (MAIR II), the purinergic receptor P2X7 or the chemokine receptor CCR5 (CD195) gave a clearly higher signal with CD11b<sup>+</sup> thymic cDC. The mAb recognizing Axl receptor tyrosine kinase stained subpopulations of both cDC subsets and a small fraction of around 10% of pDC (Figure 2c). Of all HLDA10 mAb considered in this chapter, only the anti-FLT3 and anti-Axl mAb showed a selective binding to thymic DC, while all other mAb bound also other thymic cell populations (compare Table 1).

### HLDA10 mAb recognizing antigen on both thymic cDC populations, but not on pDC

Twelve of the HLDA10 mAb were found to bind both thymic CD141<sup>+</sup> and CD11b<sup>+</sup> cDC populations, but not pDC (Figure 3). Expression of Clec7A (Dectin-1) could be detected exclusively on thymic cDC, and not on other thymic cell populations with all three HLDA10 mAb clones. Of these, clone GE2 (biotin-conjugated) gave a slightly stronger signal on CD11b<sup>+</sup> thymic cDC. CD1a, CD1b and CD1c were broadly expressed on various thymic cell populations, with the exception of pDC (Figure 3 and Table 1). CD1a was similarly detected with two mAb clones on the majority of both CD141<sup>+</sup> and CD11b<sup>+</sup> cDC subsets. MAb recognizing CD1b, CD1c, CD85h (ILT1) or CD101 generally gave a higher signal with CD11b<sup>+</sup> cDC compared with CD141<sup>+</sup> DC. Interestingly, in one of three thymus samples, no staining of CD101 on CD141<sup>+</sup> cDC was detectable, but still a strong expression on CD11b<sup>+</sup> DC was detected. The mAb clones CMRF-44 and CMRF-56 (recognizing both unknown antigens), and also the anti-Vimentin mAb SC5, stained subpopulations on both cDC subsets, but did not stain pDC. The marginal signal obtained with mAb directed to CD1a and CD101 on pDC was interpreted as background; further studies would be warranted to clarify this point. In summary, of the mAb discussed in this chapter, only anti-Clec7A and CD85h mAb exclusively stained thymic cDC; all other mAb also stained other thymic cell populations (Table 1).

### HLDA10 mAb preferentially recognizing antigen on a subpopulation of thymic CD11b<sup>+</sup> cDC

MAb of this category predominantly stained the CD11b<sup>+</sup> thymic cDC subset. MAb directed to CD273 (B7-DC), Clec2D, Clec4D (Dectin-3, now CD368), DC-SIGN like, the formyl peptide receptor 2 FPRL1 and the tyrosine kinase Tie-2 only bound to a subpopulation of the CD11b<sup>+</sup> subset (Figure 4). Expression of Clec5A (MDL-1), Clec13A (CD302), FDF03 (PILR $\alpha$ ) and the formyl peptide receptor 1 (FPR1) could be detected on the large majority of CD11b<sup>+</sup> cDC, but still not on the entire population. All of the mAb listed above selectively bound CD11b<sup>+</sup> DC, with the exception of of mAb directed to CD273 and Clec2D, which in addition stained thymic HLA-DR<sup>+</sup> non-DC (Table 1). Trem-2 (mAb FAB17291P) was found on almost all CD11b<sup>+</sup> cDC, barely on CD141<sup>+</sup> cDC, but also on a substantial subpopulation of pDC (Figure 4).



**Figure 1** Definition of primary human thymic DC subsets. Thymic CD123<sup>+</sup> HLA-DR<sup>+</sup> cells were defined as pDC, and CD123<sup>-</sup> lin<sup>-</sup> HLA-DR<sup>+</sup> CD11c<sup>int/high</sup> cells were defined as cDC and further subdivided into CD141<sup>+</sup> cDC and CD11b<sup>+</sup> cDC. The inset numbers represent cell frequencies of one tissue donor. Prior to DC subset gating, gates were set on live cells defined by scatter characteristics and exclusion of propidium iodide-positive events. Doublets were excluded using forward scatter (FSC) and side scatter (SSC) height versus area characteristics.

**Table 1** Reactivity of HLDA10 mAb to human thymic DC populations and other thymic cells

Antigen	Clone	Code	Label	cDC		pDC	HLADR <sup>+</sup> non-DC	HLADR <sup>neg</sup> thymocytes
				CD141	CD11b			
Axl	FAB154P	10-50	PE	++	++	-/+	-	-
B7-H4	MIH43	10-64	PE	-	-	-	-	-
Calreticulin	FMU-CRT-2	10-23	None	-	+	-	-	-
	FMU-CRT-8	10-29	None	-	-/+	-	-	-
	FMU-CRT-17	10-42	None (IgM)	-	-	-	-	-
CD1a	619	10-10	None	+++	+++	-	+++	+++
	010e	10-03	None	+++	+++	-	+++	+++
CD1b	O249	10-18	None	++	+++	-	++	+++
CD1c	L161	10-26	None	+++	+++	-	+++	+++
CD85g (ILT7)	17G10.2	10-66	PE	-	-	++++	-	-
CD85h (ILT1)	24	10-74	PE	++	+++	-	-	-
CD101	BB27	10-34	None	++	+++	-	-	+ / ++
CD135 (Flt-3/Flk-2)	FAB812P	10-15	PE	+++	++	+++	-	-
CD195 (CCR5)	HEK/1/85	10-76	PE	++	++++	++++	++	*
CD245	DY12	10-43	None	++	++	+	-/+	-/+
	DY35	10-48	None	-/+	+	-	-	-
CD273, B7-DC	ANC8D12	10-61	Bio	-	++	-	+	-
Clec2D/OCIL	FAB3480P	10-06	PE	-	++	-	+	-
Clec4a (DCIR)	111F8.04	10-71	FITC	-	+++	++	-/+	-
	9E8	10-72	PE	+++	++++	+++	++	+
Clec4D (Dectin-3)	FAB1748P	10-13	PE	+++	++++	+++	++	+
	9B9	10-78	PE	-	++	-	-	-
	FAB2806P	10-21	PE	-	++	-	-	-
Clec5A/MDL-1	FAB238P	10-28	PE	-	+++	-	-	-
Clec5C/NKp80	FAB1900P	10-31	PE	-	-	-	-	*
Clec7A (Dectin-1)	15 E 2	10-79	PE	++++	++++	-	-	-
	FAB1859P	10-35	PE	++++	++++	-	-	-
Clec8A/LOX-1	GE2	10-01	Bio	++	+++	-	-	-
	FAB1798P	10-40	PE	-	+	+	+	-
Clec9A (DNGR1)	8F9	10-02	None	+++	-	-	-	-
	8F9	10-65	PE	++++	-	-	-	-
	9A11	10-09	Bio	++++	-	-	-	-
	FAB6049P	10-45	PE	++++	++	-	-	-
Clec12A	50C1	10-73	PE	++++	++++	+++	-	+
	HB3	10-17	None	++++	+++	++	-	-/+
Clec13A/CD302	FAB2946P	10-51	PE	++++	++++	+++	-	-
	FAB637P	10-54	PE	-	+++	-	-	-
Clec14A	FAB7436P	10-57	PE	+	-	-	-	-
DC-SIGN like	118A8.05	10-83	FITC	-	++	-	-	-
DORA	104A10.01	10-77	FITC	-	-	-	-	-
FAT1 cadherin	FMU-FAT1-7	10-16	None	-	+	-	-	-
	FMU-FAT-6	10-08	None	-	-/+	-	-	-
FDF03	36H2	10-84	FITC	-	+++	-	-	-
FPR1	FAB3744P	10-47	PE	-	+++	-	-	-
FPRL1/FPRL2	FAB3479P	10-36	PE	-	++	-	-	-
GARP	ANC10G10	10-63	Bio	-	-	-	-	-
	ANC8C9	10-62	Bio	-	-	-	-	+
IL-1RAcP	AY19	10-53	None	++	+++	+	++	+++
IL-13 Ra2	FMU-IL-13RA2-14	10-41	None	-	++	-	-	-
	FMU-IL-13RA2-7	10-30	None	-	+	-	-	-
	FMU-IL-13RA2-8	10-37	None	-	+	-	-/+	-
LPAP	CL3	10-04	None	-	-/+	-	-	-
	CL4	10-11	None	-	-/+	-	-	-
	CL7	10-19	None	-	++	-	+	-
MAIR II	TX45	10-80	PE	+	++++	++	+	-
P2X7	L4	10-70	None	+	+++	++	++	++
Tetanus toxoid	CMRF-81	10-85	Bio	-	-	-	-	-

Table 1 (Continued)

Antigen	Clone	Code	Label	cDC		pDC	HLADR <sup>+</sup> non-DC	HLADR <sup>neg</sup> thymocytes
				CD141	CD11b			
Tie-2	FAB3131P	10-56	PE	-	++	-	-	-
TIM-1	1D12	10-67	PE	-	-/+	-	-	-
	FAB1750P	10-14	PE	-	++	-	-	-
Tim-3	F38-2E2	10-75	PE	++++	++++	+++	++	+
	FAB2365P	10-24	PE	++++	++++	++++	++	++
Tim-4	9F4	10-81	PE	-	-	-	-	-
Trem-2	FAB17291P	10-07	PE	-	+++	++	-	+
TSLP-R	1B4	10-68	PE	-	-	+	-	+
ULBP-3	FAB1517P	10-52	PE	+	+	+	+	++
Unknown	BGA69	10-38	None	+	+	-	-	-
Unknown	CMRF-44	10-82	None (IgM)	+	++	-	+++	-
Unknown	CMRF-56	10-69	None	++	+++	-	++++	+
Unknown	MDR64	10-59	None	-	-/+	-	-	-
Vimentin	SC5	10-55	None (IgM)	+	++	-	+++	-

Abbreviations: cDC, conventional dendritic cells; DC, dendritic cells; FITC, fluorescein isothiocyanate; HLDA10, 10th Human Leukocyte Differentiation Antigens workshop; mAb, monoclonal antibody; pDC, plasmacytoid dendritic cells; PE, phycoerythrin.

- binding of the mAb was similar to negative control.

\*a distinct subpopulation of 5-10% was stained by the mAb.

-/+ 10-25% of the respective cell subset was recognized/shifted by the mAb in one or two individuals only.

+ 10-25% of the respective cell subset was recognized/shifted by the mAb.

++ 25-60% of the cell subset was recognized/shifted by the mAb.

+++ 60-95% of the cell subset was recognized/shifted by the mAb.

++++ 95-100% of the cell subset was recognized the mAb.

Values represent the average of three thymus donors.

#### HLDA10 mAb recognizing antigen expressed exclusively on thymic CD141<sup>+</sup> cDC

Mab clones 8F9 or 9A11, directed to the C-type lectin Clec9A (DNCR1, now CD370), were the only mAb of the HLDA10 panel selectively recognizing CD141<sup>+</sup> thymic cDC (and no other thymic cells, Figure 5 and Table 1). The third anti-Clec9A clone FAB6049P in addition gave a signal with CD11b<sup>+</sup> cDC (Figure 5). This discrepancy needs further evaluation.

#### HLDA10 mAb recognizing antigen expressed exclusively on pDC

Mab clone 17G10.2, specific for CD85g (ILT7), exclusively recognized thymic pDC. Other thymic cell populations, including cDC, did not give any signal (Figure 5 and Table 1).

#### HLDA10 mAb giving only marginal staining with at least one DC population

Although the majority of the tested HLDA10 mAb was able to clearly detect cell surface molecules on human thymic DC, some HLDA10 mAb showed only weak staining on at least one human thymic DC subset; mAb recognizing TIM-1 (HAVCR1, now CD365), TIM-4, Clec8A (LOX-1), Clec14A and ULBP-3 belonged to this group (Figure 6, upper row). Expression of the TSLP-R was detected only on minor subpopulations of both thymic cDC subsets (5-10% of each subset) and on approximately 20% of pDC (Figure 6, upper panels). For a number of HLDA10 mAb provided as unlabeled IgG antibodies (CD245, IL-13 Ra2, LPAP (lymphocyte phosphatase-associated) or FAT1 cadherin), specific binding could not easily be distinguished from background signal, especially on CD11b<sup>+</sup> cDC (Figure 6, lower panels).

#### HLDA10 mAb not recognizing human thymic cells

The provided HLDA10 mAb directed to B7-H4 (clone MIH43), DORA (104A10.01), GARP (ANC10G10, ANC8C9), Tetanus toxoid

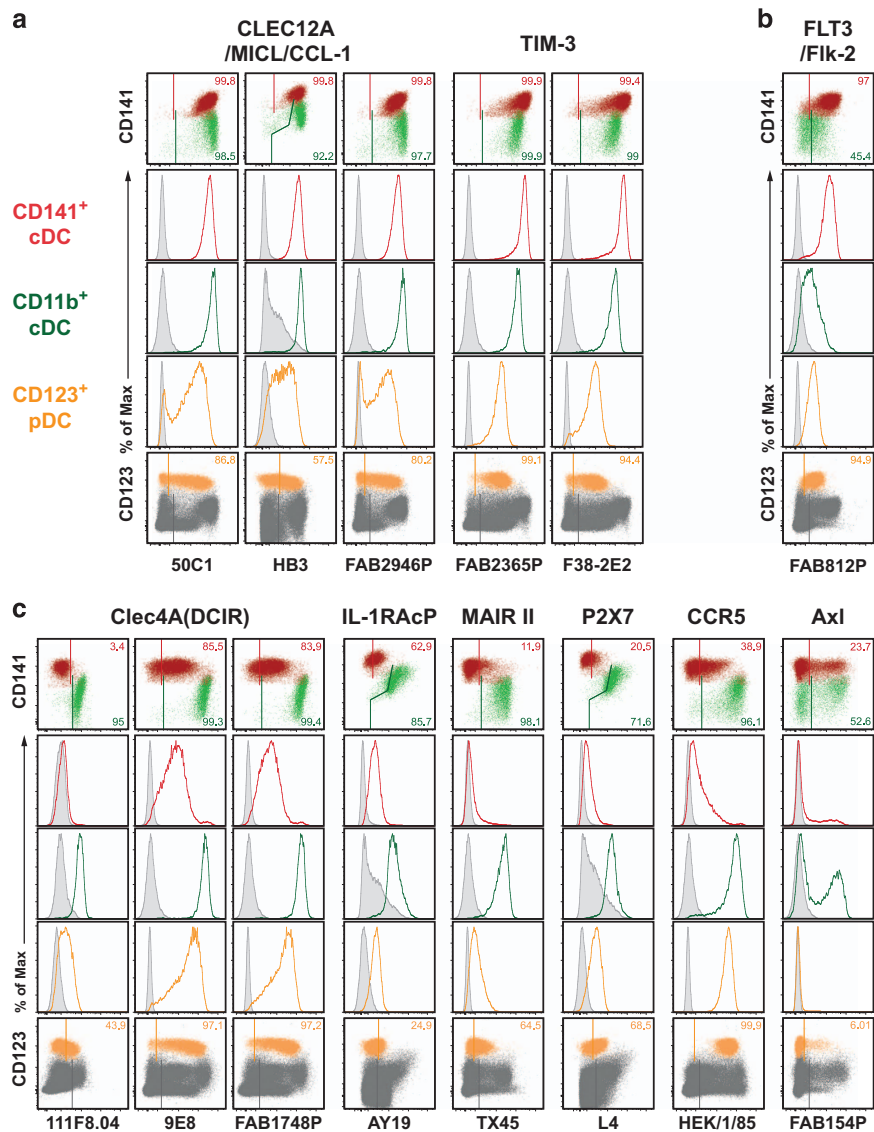
(CMRF-81) and the mAb MDR64 (flow cytometry data not shown) did not stain any of the tested Nycodenz-enriched thymic cell populations. The anti-Clec5C mAb FAB1900P did stain HLA-DR<sup>neg</sup> thymocytes, but failed to bind to any of the primary thymic DC subsets (see Table 1).

#### Discrepancies in the staining pattern of HLDA10 mAb

In most cases, different HLDA10 mAb clones directed to the same antigen gave similar staining results. Nevertheless, a few exceptions were seen. For example, binding of the anti-Clec4A mAb clones 9E8 and FAB1748P (coupled to PE) resulted in a very bright staining of CD11b<sup>+</sup> cDC and pDC, and also gave a clearly detectable signal with CD141<sup>+</sup> cDC, whereas clone 111F8.04 (conjugated to fluorescein isothiocyanate) did not stain CD141<sup>+</sup> cDC; in this case, the observed difference most likely results from a lower affinity of clone 111F8.04 and/or a lower brightness of fluorescein isothiocyanate (Figure 2c). The differences in the staining characteristics of the various Clec9A mAb clones (Figure 5) were discussed in the respective section. The anti-Clec12A clone FAB2946P selectively stained DC and no other tested cell population, while clones 50C1 and HB3 also recognized a small population of HLA-DR<sup>neg</sup> thymocytes. The unlabeled IgG mAb FMU-CRT-2 and FMU-CRT-8, which should recognize calreticulin, slightly stained CD11b<sup>+</sup> DC, but the anti-calreticulin IgM clone FMU-CRT-17 did not stain any thymic cell population. Whether the listed discrepancies were due to differences in the avidity of given mAb clones for their antigen, resulted from the use of different secondary reagents, or simply reflected higher unspecific binding, require further evaluation.

#### DISCUSSION

All results provided here were obtained with DC isolated by mechanical and enzymatical disruption of thymic tissue obtained from three children undergoing surgery for congenital heart defects at



**Figure 2** HLDA10 mAb binding to both thymic cDC and pDC. A larger number of HLDA10 mAb recognized both cDC subsets and pDC, and to a variable extent also other thymic cell populations. Dot plots show stainings as overlays of CD141<sup>+</sup> (red) versus CD11b<sup>+</sup> (green) cDC (top row), or pDC (orange) within total thymocytes (dark gray) (bottom row). Inset numbers give the proportion of the respective DC populations stained. Gates were set according to background controls (FMO for fluorophore-labeled mAb, secondary reagent controls for IgM or biotinylated mAb or isotype controls for unlabeled IgG mAb). Histogram overlays (middle rows) show signals of the panel mAb obtained with the color-coded thymic DC populations (open histograms) in comparison with the background staining (filled-in gray). The molecules recognized by these mAb could be grouped into being (a) similarly expressed on both cDC subsets, (b) expressed to a higher degree on CD141<sup>+</sup> cDC and (c) expressed to a higher degree on CD11b<sup>+</sup> cDC. The reactivity with other thymic cell populations can be seen in Table 1. Shown are representative data from one out of three thymic tissues.

the age of 2–8 months; these infants were otherwise healthy. Nycodenz enrichment, performed because of the low frequency of DC in the thymus, allowed to analyze all DC subsets (pDC, CD141<sup>+</sup> cDC and CD11b<sup>+</sup> cDC) with the HLDA10 mAb in parallel. At the same time, this enrichment reduced the proportion of thymic lymphocytes (developing T cells, B and NK cells, all defined via lineage markers) in the preparation. The Nycodenz-enriched cell preparation also contained HLA-DR<sup>+</sup> cells which were negative for CD11c, CD141 and CD123, and were thus not regarded as DC. As a general caveat, we cannot exclude that some small, higher-density thymic population was lost during the DC isolation and enrichment procedure used.

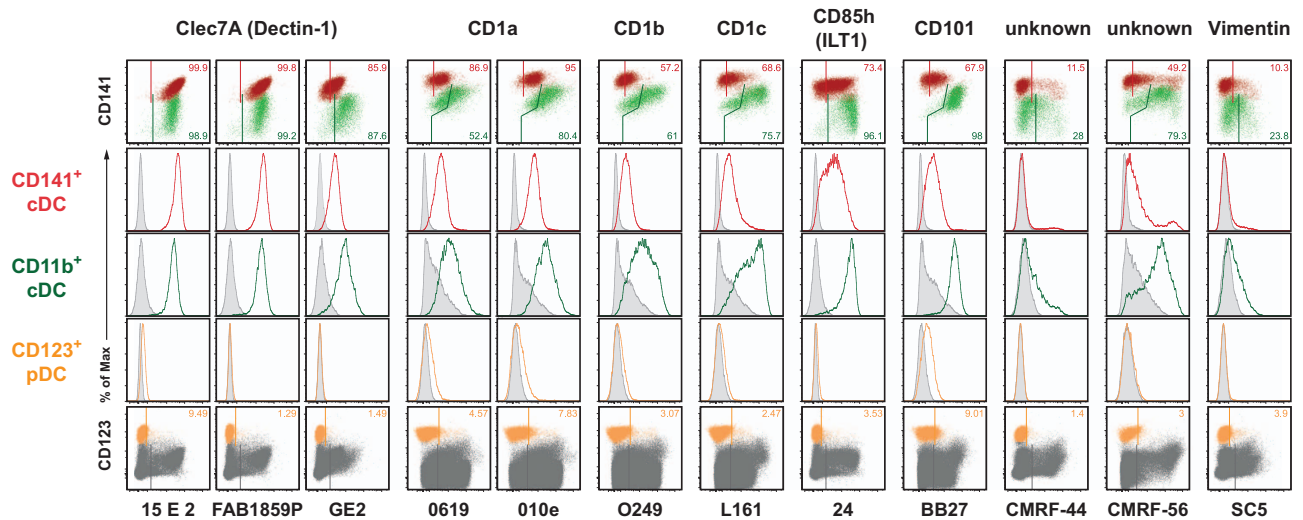
On the basis of the characterization of thymic DC subsets by Vandenabeele *et al.*,<sup>7</sup> expression of CD123 was used to define pDC. As

described in the introduction, CD141 and CD11b were used to subdivide thymic cDC, which were defined as CD123<sup>neg</sup> lineage<sup>neg</sup> HLA-DR<sup>+</sup> CD11c<sup>int/high</sup> cells. It has been very recently shown that this phenotypic subdivision also reflects functional differences between these two human thymic cDC subsets.<sup>13</sup>

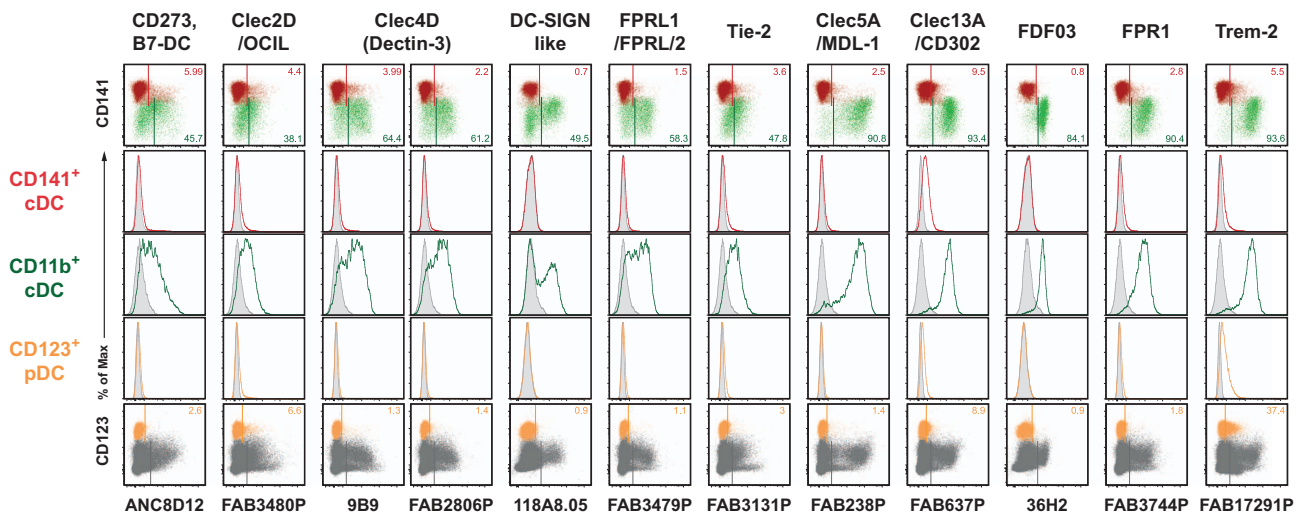
All flow cytometry stainings described in this report were carried out using a concentration of the tested mAb recommended by the HLDA10 workshop. This concentration may not give optimal stainings in terms of signal-to-noise ratio and specificity in all instances. Because of this technical limitation, a more detailed analysis of antigens that are apparently expressed at low levels is warranted.

Furthermore, we cannot be certain whether the expression pattern obtained with the HLDA10 panel on thymic DC from infants will





**Figure 3** HLDA10 mAb binding to both thymic cDC subsets but not to pDC. HLDA10 mAb reacting with cDC but not pDC. Dot plots show stainings as overlays of CD141<sup>+</sup> (red) versus CD11b<sup>+</sup> (green) cDC (top row), or pDC (orange) within total thymocytes (dark gray) (bottom row). Inset numbers give the proportion of the respective DC populations stained. Gates were set according to background controls (FMO for fluorophore-labeled mAb, secondary reagent controls for IgM or biotinylated mAb or isotype controls for unlabeled IgG mAb). Histogram overlays (middle rows) show signals of the panel mAb obtained with the color-coded thymic DC populations (open histograms) in comparison with the background staining (filled-in gray). The minimal signals with mAb to CD1a, CD1b, CD1c and CD101 on pDC were regarded as background. The reactivity with other thymic cell populations can be seen in Table 1. Shown are representative data from one out of three thymic tissues.



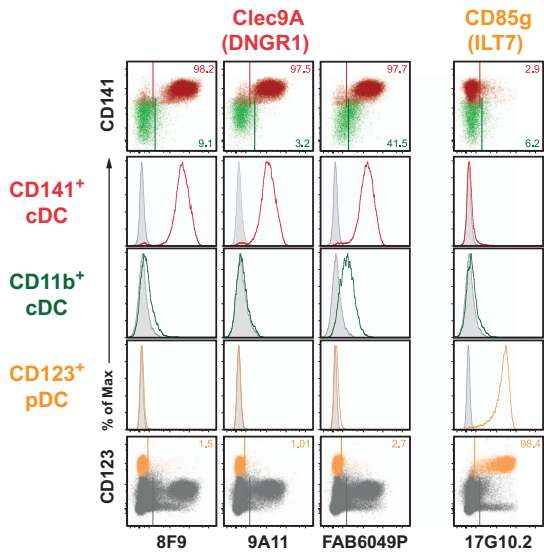
**Figure 4** HLDA10 mAb with preferential binding to CD11b<sup>+</sup> cDC. A number of panel mAb recognized mainly or selectively molecules on CD11b<sup>+</sup> cDC. Dot plots show stainings as overlays of CD141<sup>+</sup> (red) versus CD11b<sup>+</sup> (green) cDC (top row), or pDC (orange) within total thymocytes (dark gray) (bottom row). Inset numbers give the proportion of the respective DC populations stained. Gates were set according to background controls (FMO for fluorophore-labeled mAb, secondary reagent controls for IgM or biotinylated mAb or isotype controls for unlabeled IgG mAb). Histogram overlays (middle rows) show signals of the panel mAb obtained with the color-coded thymic DC populations (open histograms) in comparison with the background staining (filled-in gray). The reactivity with other thymic cell populations can be seen in Table 1. Shown are representative data from one out of three thymic tissues.

completely match the pattern on adult thymocytes. In this context, however, it is interesting to note that the observed expression characteristics of several HLDA10 antigens resembled the pattern described for peripheral DC in adults (see references below).

A substantial proportion of the tested HLDA10 mAb not only bound thymic DC, but also other cells. This can be concluded for mAb recognizing CD1a, CD1b and CD1c, CD101, CD195 (CCR5), IL-1RacP, P2X7, TIM-3, Vimentin, and the unknown antigens recognized by mAb clones CMRF-44 and CMRF-56. Interestingly, CD1c, commonly used to demarcate peripheral CD141<sup>neg</sup> cDC,<sup>8,9</sup>

could not be used for this purpose with thymic tissue. As exemplified with HLDA10 clone L161, a bright CD1c signal was obtained on thymic CD141<sup>neg</sup> CD11b<sup>+</sup> cDC, but also CD141<sup>+</sup> cDC were positive, as was the majority of all other tested thymic cell populations.

Expression of the thymic stromal lymphopoietin receptor (TSLP-R), which (by binding TSLP) enables thymic DC to induce regulatory T cells,<sup>16</sup> was surprisingly found only on small subpopulations (5–10%) of both CD141<sup>+</sup> and CD11b<sup>+</sup> cDC, and in addition on approximately 20% of pDC. Recently, Martinez *et al.*<sup>13</sup> obtained with the same mAb clone a bright staining of TSLP-R on the majority of BDCA3<sup>high</sup>



**Figure 5** HLDA10 mAb with selective recognition of thymic CD141<sup>+</sup> DC or pDC. Mab of the HLDA10 panel reacting with only one DC subset. Dot plots show stainings as overlays of CD141<sup>+</sup> (red) versus CD11b<sup>+</sup> (green) cDC (top row), or pDC (orange) within total thymocytes (dark gray) (bottom row). Inset numbers give the proportion of the respective DC populations stained. Gates were set according to background controls (FMO for fluorophore-labeled mAb, secondary reagent controls for IgM or biotinylated mAb or isotype controls for unlabeled IgG mAb). Histogram overlays (middle rows) show stainings of the HLDA10 mAb on the color-coded thymic DC populations (open histograms) in comparison with the background staining (filled-in gray). MAb 8F9 and 9A11 directed to Clec9A/DNGR1 selectively recognized CD141<sup>+</sup> DC, while the third clone FAB6049P exhibited additional reactivity with CD11b<sup>+</sup> cDC. One mAb (clone 17G10.2) directed to CD85g selectively stained pDC. Shown are representative data from one out of three thymic tissues.

(= CD141<sup>+</sup>) thymic cDC. Differences in the isolation procedure of thymic DC may only partially explain this discrepancy. Although tissue digestion (used by us) may have destroyed epitopes for mAb binding, isolation without digestion (used by Martinez *et al.*) led to a strongly reduced frequency of all thymic DC subsets, and may have resulted in the relative absence of TSLP-R<sup>neg</sup> DC in the preparation. Of note, TSLP-R expression was also not detectable on the corresponding human peripheral blood DC subsets isolated without any digestion step<sup>17</sup> (HLDA9, same mAb clone).

The staining of another set of analyzed HLDA10 mAb can be regarded as restricted to thymic cDC and pDC, because all other thymic populations were negative. Among these were two receptor tyrosine kinases, FLT3 (CD135) and Axl. In the bone marrow, FLT3 is expressed on hematopoietic progenitor cells, and is described to be required for DC development.<sup>18</sup> In the spleen, FLT3 is expressed only on DC at steady state.<sup>19</sup> In the human thymus, we were able to detect FLT3 expression only on DC subsets, with nearly all CD141<sup>+</sup> cDC and pDC being positive. As recently reported for peripheral blood DC and lung DC,<sup>10</sup> also, thymic CD141<sup>+</sup> cDC showed significantly higher FLT3 levels compared with CD141<sup>neg</sup> CD11b<sup>+</sup> (or CD1c<sup>+</sup>) DC. Axl kinase has been implicated in the clearance of apoptotic cells and in the facilitation of antigen cross-presentation by DC.<sup>20,21</sup> Expression of Axl on human thymic DC was not homogenous in that only subpopulations of the various subsets stained positive (approximately 30% of CD141<sup>+</sup>, 60% of CD11b<sup>+</sup> and around 5–10% of pDC).

Clec7A (Dectin-1, now CD369) appears to be exclusively expressed on total thymic cDC, and this is consistent with previous reports of

preferential expression by mouse and human DC.<sup>22–24</sup> We found CD85h (ILT1, a member of the Ig-like transcripts family) to be similarly restricted to thymic cDC, which is congruent with a previous analysis of peripheral blood DC.<sup>25</sup> Expression of CD85h was clearly higher on CD11b<sup>+</sup> cDC as compared with CD141<sup>+</sup> cDC.

The HLDA10 mAb recognizing Clec4D, Clec5A, Clec13A, DC-SIGN like, FDF03, FPR1 and FPRL1 were only detected on subpopulations of thymic CD11b<sup>+</sup> cDC, so these molecules may be either candidates for the definition of new subpopulations of CD11b<sup>+</sup> cDC, or they simply mark different activation/differentiation states within the same DC population. To address this issue, further experiments analyzing the transcriptional profile or specific functions of separated subpopulations will be required.

Few of the HLDA10 mAb tested specifically recognized only one thymic DC subset. CD85g (ILT7) was detected exclusively on thymic pDC, as already described for peripheral blood and tonsils.<sup>26–28</sup> MAb recognizing Clec5A, Clec13A, FDF03 and FPR1 all stained a large majority of CD11b<sup>+</sup> thymic cDC (each 60–90%, with variation between donors) and no other thymic population. However, none of the HLDA10 mAb was able to exclusively stain the entire CD11b<sup>+</sup> cDC subset. The only mAb in the HLDA10 panel specifically recognizing CD141<sup>+</sup> cDC were the clones 8F9 and 9A11, directed to Clec9A (now CD370). Clec9A/DNGR1 was previously shown to be expressed on cross-presenting DC in the mouse<sup>29</sup> and on the homologous CD141<sup>+</sup> cDC in human peripheral blood and various other tissues.<sup>10,30,31</sup> The third Clec9A-specific clone FAB6049P, however, also exhibited significant binding to CD11b<sup>+</sup> DC, and this issue needs further clarification.

In the human, Clec9A thus is a clear candidate for a lineage marker of cross-presenting DC. Other molecules known to be specifically and exclusively expressed on human CD141<sup>+</sup> cDC (and their rhesus macaque and mouse homologs) are the chemokine receptor XCR1 and the cell adhesion molecule CADM1. These three molecules can today be regarded as the best markers for cross-presenting DC in various tissues across various species.<sup>12,14,32–35</sup>

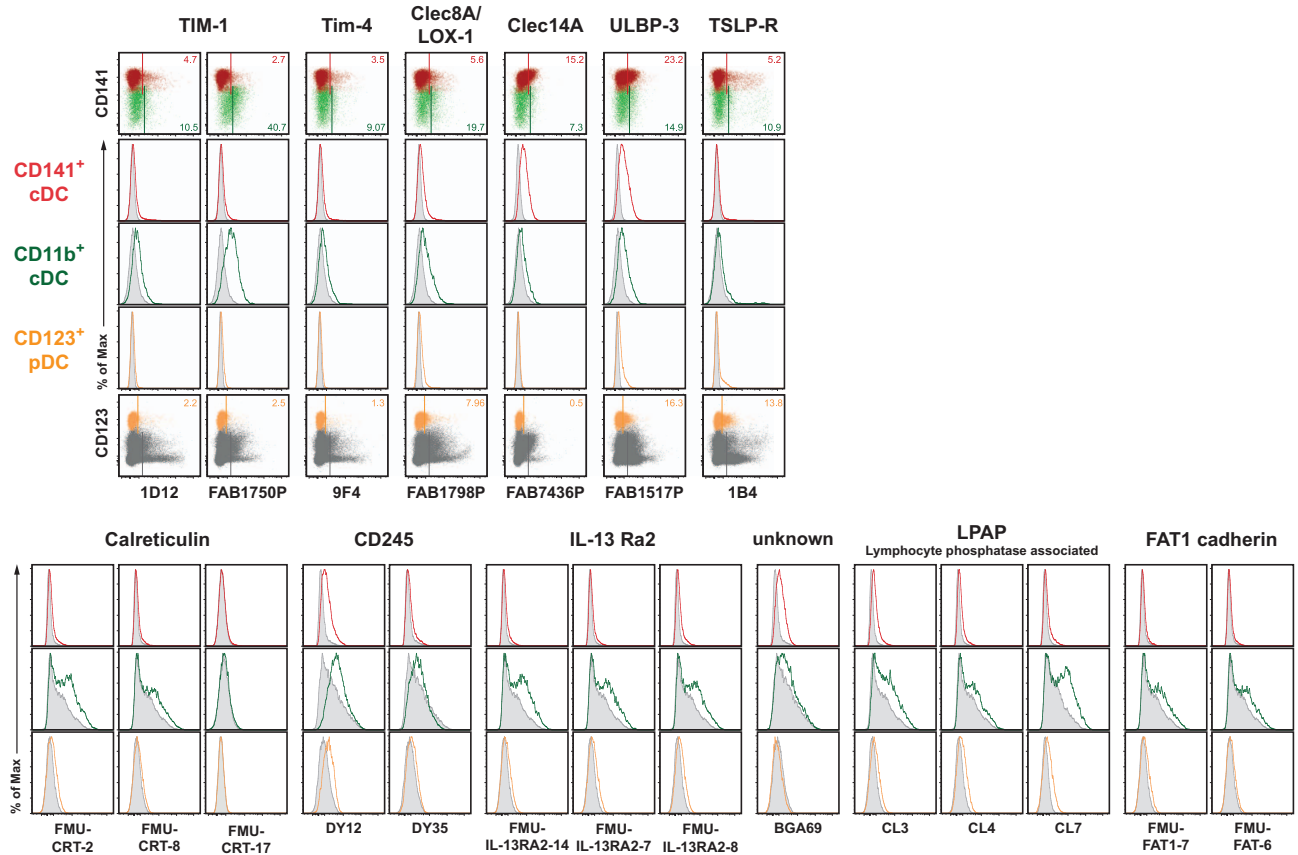
## METHODS

### Cell isolation

Thymic tissue was obtained from newborns and infants up to the age of 8 months undergoing cardiac surgery for congenital heart defects and having no additional health issues. The analysis of DC in thymic tissues was approved by the Charité Ethics Committee. Individual tissues were used only after informed consent of the parents. Thymic tissue was cut into small pieces and digested for 20 min with collagenase D (500 µg ml<sup>-1</sup>) and DNase I (20 µg ml<sup>-1</sup>, both Roche, Mannheim, Germany) in RPMI 1640 containing 2% fetal calf serum (low endotoxin, Biochrom, Berlin, Germany) supplemented with 10 mM EDTA for the final 5 min of incubation. DC were further enriched by Nycodenz-gradient centrifugation (Nycoprep, Axis-Shield, Oslo, Norway). Cells were frozen in complete medium and 10% dimethyl sulfoxide, and stored in liquid nitrogen until use.

### Flow cytometry

Detailed information on the HLDA10 mAb can be found at <http://www.hcdm.org>. For staining of cells, test mAb were used at concentrations recommended by the HLDA10 workshop. To define the DC subsets, antibodies directed to HLA-DR, CD11c, CD123, CD141 and CD11b were used, the lineage markers CD7 (or CD3), CD19 and CD56 were used for gating out developing T lymphocytes, B cells and NK cells. Before staining, cells were pre-incubated with 1 mg ml<sup>-1</sup> human IgG (Endobulin, Baxter, Heidelberg, Germany) to block unspecific binding of mAb via the Fc-portion. After incubation with a biotinylated, unlabeled IgM, PE- or fluorescein isothiocyanate-coupled test mAb, cells were washed and stained with an antibody mix, which included the secondary reagent (PE-conjugated Streptavidin (SAV, eBioscience,



**Figure 6** HLDA10 mAb giving only marginal staining on at least one thymic DC population. MAb of the HLDA10 panel with low or unclear reactivity. Dot plots show stainings as overlays of CD141<sup>+</sup> (red) versus CD11b<sup>+</sup> (green) cDC (top row), or pDC (orange) within total thymocytes (dark gray) (bottom row). Inset numbers give the proportion of the respective DC populations stained. Gates were set according to background controls (FMO for fluorophore-labeled mAb, secondary reagent controls for IgM or biotinylated mAb or isotype controls for unlabeled IgG mAb). Histogram overlays (middle rows) show stainings of the HLDA10 mAb on the color-coded thymic DC populations (open histograms) in comparison with the background staining (filled-in gray). Shown are representative data from one out of three thymic tissues.

**Table 2** Staining scheme for testing HLDA10 mAb on primary human thymic DC

Type of test mAb	FITC	PE	PE-Cy7	APC/Cy5	A700	PacB / BV421	BV650
mAb-Bio	CD7, CD19, CD56	+ SAV-PE	CD11c	CD141	CD11b	HLA-DR	CD123
mouse IgM mAb purified	CD7, CD19, CD56	+ $\alpha$ - IgM PE	CD11c	CD141	CD11b	HLA-DR	CD123
mAb-PE	CD7, CD19, CD56	(mAb-PE)	CD11c	CD141	CD11b	HLA-DR	CD123
mAb-FITC	(mAb-FITC)	CD11b	CD11c	CD141	HLA-DR	CD3, CD19	CD123
mouse IgG mAb purified/ascites	CD7, CD19, CD56	CD11b	CD11c	+ $\alpha$ -IgG Cy5	HLA-DR	CD141	CD123

Abbreviations: APC, antigen-presenting cell; DC, dendritic cells; FITC, fluorescein isothiocyanate; HLDA10, 10th Human Leukocyte Differentiation Antigens workshop; mAb, monoclonal antibody; PE, phycoerythrin.

San Diego, CA, USA) for biotinylated mAb, or mAb Bet-2 (ATCC HB-88, ATCC, Manassas, VA, USA), for unconjugated IgM mAb (Table 2). Bound unlabeled IgG test mAb were first reacted with goat anti-mouse IgG-Cy5 (Fc $\gamma$ -specific, Dianova, Hamburg, Germany), washed, incubated with mouse gamma-globulin (Dianova) to block all free valencies of the secondary reagent, and then stained with the mAb-mix (Table 2). Background staining of unlabeled mAb was determined using the isotype control mAb MOPC-21 (IgG1, Sigma, St Louis, MO, USA), S43-10 (IgG2a<sup>36</sup>) or D3-13F1 (IgG2b, gift from K. Rajewsky). Staining was performed on ice according to standard methods.

Fluorophore-conjugated mAb recognizing human CD7 (clone CD7-6B7), CD56 (HCD56), CD11c (Bu15), CD123 (6H6) and CD141 (M80) were from BioLegend (San Diego, CA, USA), CD141 (AD5-14H12) from Miltenyi Biotec, Bergisch Gladbach, Germany. MAb recognizing human CD3 (OKT3, ATCC

CRL-8001), CD11b (OKM1, ATCC CRL-8026), CD19 (BU12<sup>37</sup>), HLA-DR (L234, ATCC HB-55) or mouse IgM (Bet-2) were purified from hybridoma supernatants and coupled to PE, Pacific Blue or Alexa Fluor 700 (Molecular Probes, Eugene, OR, USA) by standard procedures.

For flow cytometry, analysis gates were set on live cells defined by scatter characteristics and exclusion of propidium iodide-positive events. Doublets were excluded using forward scatter (FSC) and side scatter (SSC) height versus area characteristics. Data were acquired on a LSR-Fortessa flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.



## ACKNOWLEDGEMENTS

This work was supported by DFG grant Kr827/18–1 to RAK.

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