

Research article

Flow cytometric assessment of the reactivity of a panel of monoclonal antibodies (mAb) against two populations of human dendritic cells (DC)

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Published: 31 July 2001

Received: 31 March 2001

BMC Immunology 2001, 2:6

Accepted: 31 July 2001

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Abstract

Background: The identification of antigens on human DC has been a very difficult and elusive task because of the lack of appropriate reagents. Therefore, we evaluated by flow cytometry a panel of mAb that recognize antigens on human DC, aiming to determine the kinetics of DC antigen expression at 7, 14, 21 and 28 days in (i) Dermal DC like cells (Mo-DC) and (ii) Langerhans cell like DC (Mo-LC). In addition we aimed to identify markers for DC subpopulations.

Results: It was found at day 7, that mAb BG6, HP-F1, BU10, RFD-1, CMRF-44 recognized <20% of Mo-DC. In contrast, 7H5, ZM3.8, CD1b/c, 55K-2, MMR1.16, MMR190.BB3 and L25 reacted with >50% of Mo-DC. Moreover, 7H5, ZM3.8, CMRF-56, CD1b/c, 55K-2, MMR1.16, MMR190.BB3 and L25 showed increased MFI reactivity against Mo-DC. mAb BG6, BU10 and CMRF-44 recognized <20% Mo-LC while RFD-1 reacted with 21% of Mo-LC. In contrast, HP-F1 showed 87% of Mo-LC positive. Also, 7H5, ZM3.8, RFD-7, MR15-2, CD1b/c, 55K-2, MMR1.16, MMR190.BB3 and L25 reacted with >50% of Mo-LC. The increase in % of positive cells was paralleled by MFI increases.

At day 14, fourteen mAb recognized >50% of the Mo-DC, while five recognized 20-50% of Mo-DC. BG6 reacted with 7% of the Mo-DC. Nineteen mAb recognized >48% of Mo-LC while BG6 had negative reactivity.

At day 21 and 28, all mAb reacted with >20% of Mo-DC and yielded a significant MFI with Mo-DC. Also nineteen mAb yielded significant MFI with Mo-LC while RFD-7 did not.

Conclusions: The immunophenotyping assays demonstrated differences between the two DC populations as well as variations in the reactivity of the mAb at diverse time points, suggesting the existence of subpopulations within the Mo-DC and Mo-LC.

Background

Dendritic cells (DC) are a complex group of mainly bone marrow derived cells that play an important role in the afferent branch of the immune response [1]. However,

DC represents only a minute subpopulation of the peripheral blood mononuclear cells (PBMC), as well as of bulk cellular populations of the lung, intestine, genitourinary tissue, and lymphoid tissue. DC also has been

found in the epidermis, dermis and mucous membranes and constituting about 2% of the total cellular population of the human epidermis [2,3]. The Langerhans cells (LC) are a skin derived-DC, that have the capability to travel to the regional lymphoid organs after take up of antigen and undergo there an activation/maturation step. Thereafter, LC interacts and activates T cells. Because of such significant capability to take up soluble antigens, process and present them to responder cells in the lymphoid tissues in the context of the restricted MHC pathway, LC have been considered one of the most important elements in the afferent arm of the immune response [1-4].

Recent, successful efforts to generate DC from PBMC derived monocytes or from CD34 blood precursors by utilizing GM-CSF and IL-4, as well as GM-CSF and/or TNF, has enabled us to obtain PBMC derived DC (Mo-DC) [5-8]. In addition, an approach has been developed to generate LC from isolated monocytes (Mo-LC) [9].

Despite the successful efforts in the generation of DC from blood precursors, the characterization of surface markers on human DC has been a very difficult and elusive task because the lack of appropriate reagents with high specificity for DC identification [4]. However, some molecules whose genes recently have been cloned and sequenced (e.g. CD83, DEC-205) have been found strongly associated with DC [5,6]. In addition, a panel of monoclonal antibodies (e.g. CMRF-44) that recognize molecules on DC has been raised [7]. There is a growing need for cluster and establishment of a common and comprehensive nomenclature for such DC associated molecules, as well as to clarify and define the lineage(s) of DC and the existence of DC subsets. These developments have prompted the set up of diverse approaches that evaluated the reactivity of a group of mAb against populations of DC [10-29]. Therefore and within the scope of this study, we set up a flow cytometry approach and evaluated a panel of 20 mAb against two populations of DC aiming to determine the kinetics of expression of antigens on DC at diverse intervals of time and the likelihood to identify markers for DC subsets [12-29].

Results and Discussion

Flow cytometric assessments of monoclonal antibodies (mAb) reactivity against dendritic cells (DC) was undertaken by measurement of the % of reactivity (Figure 1,2,3,4) and mean fluorescent intensity (MFI) of two populations of DC at 7, 14, 21 and 28 days (Table 1). At day 7 (Figure 1), the flow cytometry evaluation showed that, antibodies 70011 (BG6), 70194 (HP-F1 (CD85i)), 70293 (BU10), 70511 (RFD-1) and 70375 (CMRF-44) recognized less than 20 % of Mo-DC. In contrast, 70345 (7H5 (CD85a)), 70358 (ZM3.8 (CD85j)), 70807 (CD1b/c), 70806 (55K-2 fascin), 70776 (MMR1.16 (CD206)),

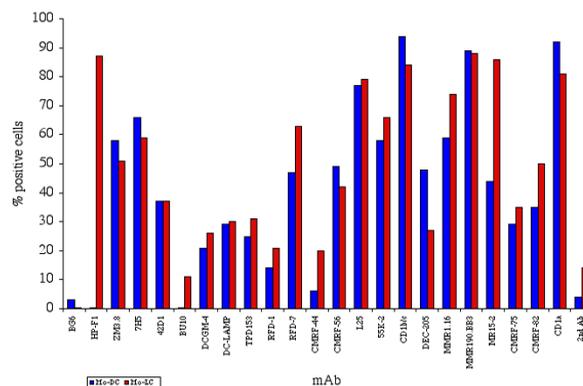


Figure 1
Percentage of positive cells detected by mAb against Mo-DC and LC-DC after 7 days of culture

70767 (MMR190.BB3 (CD206)) and 70772 (L25) reacted with more than 50% of Mo-DC. Moreover, 70345 (7H5 (CD85a)), 70358 (ZM3.8 (CD85j)), 70376 (CMRF-56), 70807 (CD1b/c), 70806 (55K-2 fascin), 70776 (MMR1.16 (CD206)), 70767 (MMR190.BB3 (CD206)) and 70772 (L25) also showed increase MFI reactivity against Mo-DC (Table 1).

The mAb 70011 (BG6), 70293 (BU10) and 70375 (CMRF-44) recognized less than 20% Mo-LC while 70511 reacted with only 21% of the cells. In contrast, 70194 (HP-F1 (CD85i)) showed that 87% of Mo-LCs were positive. Also, 70345 (7H5 (CD85a)), 70358 (ZM3.8 (CD85j)), 70512 (RFD-7), 70802 (MR15-2 (CD205)), 70807 (CD1b/c), 70806 (55K-2 fascin), 70776 (MMR1.16 (CD206)), 70767 (MMR190.BB3 (CD206)), 70772 (L25) reacted with more than 50% of Mo-LC. The increase in % of positive cells was also paralleled by MFI increases (Table 1).

At day 14 (Figure 2), 14 mAb recognized more than 50% of the Mo-DC, while 70427 (DCGM-4 (CD207)), 70451 (TPD153), 70511 (RFD-1), 70512 (RFD-7) and 70375 (CMRF-44) recognized between 20 and 50% of Mo-DC. 70011 (BG6) reacted with 7% of the Mo-DC. The reactivity of 70011 (BG6) against Mo-LC was indistinguishable from the background while the other nineteen mAb recognized more than 48% of Mo-LC. At day 21 and 28, all the mAb reacted with more than 20% of Mo-DC (Figure 3 and 4). Also, the mAb yielded a significant MFI with Mo-DC (Table 1). In addition, nineteen mAb yielded significant MFI with Mo-LC whilst only 70512 (RFD-7) did not. (Table 1) At day 21 (Figure 3), seventeen mAb reacted with more than 50% of the Mo-DC, only 70346 (42D1

Table 1: MFI of mAb reactivity against HDC

	Day 7		Day 14		Day 21		Day 28	
	Mo-DC	Mo-LC	Mo-DC	Mo-LC	Mo-DC	Mo-LC	Mo-DC	Mo-LC
BG6	72	57	81	149	637	256	644	1100
HP-F1	48	274	160	199	717	310	615	1252
ZM3.8	180	155	332	305	515	226	747	1025
7H5	176	153	366	402	387	221	382	859
42D1	138	129	175	157	278	150	291	562
BU10	50	97	171	172	296	161	392	320
DCGM-4	115	125	177	193	570	288	608	1317
DC-LAMP	148	126	296	205	601	252	567	712
TPD153	124	141	189	198	482	510	657	808
RFD-1	102	113	161	175	374	193	376	712
RFD-7	150	167	209	621	165	114	168	158
CMRF-44	79	111	160	185	348	152	339	115
CMRF-56	193	200	209	203	977	421	974	2232
L25	334	353	279	336	866	890	792	1430
55K-2	237	228	373	215	1029	526	1044	2020
CD1b/c	789	515	392	398	408	234	384	654
DEC-205	162	127	227	171	548	281	430	915
MMR1.16	194	265	319	281	570	272	656	1147
MMR190.BB3	716	570	702	778	881	447	1019	1917
MR15-2	151	278	547	520	721	476	588	817
CMRF-75	155	209	266	234	432	186	403	507
CMRF-82	153	182	340	232	760	357	946	1420
CD1a	801	493	423	603	443	232	354	434
2nd Ab	80	106	121	165	97	81	85	105

(CD85f)), 70511 (RFD-1) and 70512 (RFD-7) were below 50%. In contrast, only seven mAb reacted with more than 50% Mo-LC but only 70512 (RFD-7) reacted with less than 20% Mo-LC. At day 28 (Figure 4), sixteen mAb reacted with more than 50% Mo-DC. The mAb 70346 (42D1 (CD85f)), 70511 (RFD-1), 70512 (RFD-7) and 70808 (DEC-205 (CD205)) were below 50% while 70375 (CMRF-44) reacted with less than 20% of Mo-LC. Seventeen mAb reacted with more than 50% of Mo-LC.

The simultaneous evaluation on Mo-DC and Mo-LC of FSC (flow cytometric assessment of size) and mAb reactivity at four time points demonstrated the presence of significant variability within the Mo-DC and Mo-LC populations (Figure 5). At day seven, there was several mAb that showed strong reactivity and can be grouped together (Figure 5 panel C mAb-MMR.190.BB3 and panel E mAb-L25). In addition there was another group of mAb with an intermediate level of reactivity that can be grouped together (Figure 5 panel D mAb-7H5 and panel F mAb-ZM-38). However, some mAb showed no reactivity at day seventh (Figure 5 panel A mAb-BG6) compared with the negative control (Figure 5 panel B-Second Ab). In addition, variations in the reactivity of the mAb at di-

verse time points were found. Moreover, at day seven it was found that mAb HP-F1 was negative for Mo-DC and positive for Mo-LC, thus discriminating Mo-DC from Mo-LC. Overall, this set of results demonstrates the existence of antigenic differences between Mo-DC and Mo-LC, even though there was only a single difference in the cytokine mixture utilized. Moreover, within the panel analyzed, it was found that at day 7, there is a mAb that showed reactivity only against Mo-DC with high FSC (mAb-TPD153) or against a fraction of Mo-DC (mAb-DC-LAMP, mAb-55K-2). These results point toward the existence of subsets within the Mo-DC and Mo-LC populations.

Of note, the studies on the reactivity of two set of mAb (mannose receptor and immunoglobulin-like transcript molecules) raised very interesting considerations about the role of these antigens in the functionality of DC.

This study showed that DC expressed significant amounts of two lectin-type receptors: the mannose receptor (MMR) detected by the mAbs MR 15-2 (CD206), MMR1.16 (CD206), and MMR190.BB3 (CD206?), and the antigen DEC-205 detected by the mAbs DEC-205

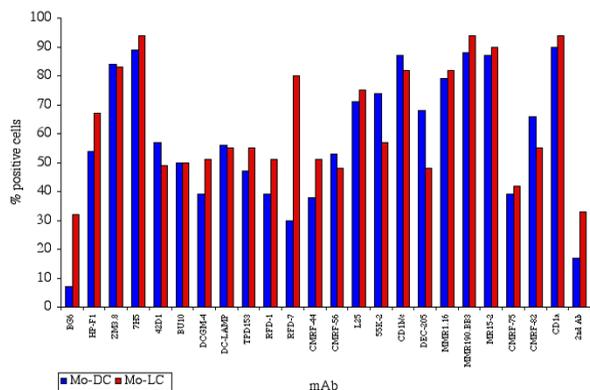


Figure 2
Percentage of positive cells detected by mAb against Mo-DC and LC-DC after 14 days of culture

(CD205) and MMRI-4 (CD205). Both molecules belong to a family of transmembrane C-type lectins. Both molecules have (i) a cysteine-rich domain, (ii) a fibronectin type II domain, (iii) multiple C-type carbohydrate recognition-like domains, (iv) a transmembrane domain and (v) a short cytoplasmic tail [6,12,24,25,27]. Both molecules the MMR [24,25,27] and DEC-205 [6,12], have been implicated in the uptake of carbohydrate-conjugated antigens by DC. MMR has been found on the surface of macrophages and its carbohydrate recognition domains mediate the endocytosis of (i) glycoconjugates containing mannose, (ii) fucose, (iii) acetylglucosamine, (iv) glucose residues, (v) microorganisms expressing

mannose or acetylglucosamine on the surface. Notably, all these terminal sugars are neither common membrane components of mammalian cells nor common components of serum proteins. Therefore, it is feasible to consider that this two lectin-type receptors: the mannose receptor (MMR) and DEC-205 may be involved in the discrimination between self and non-self antigens as well and could contribute to enhance the capability of dendritic cells to generate primary T cell responses against infectious agents and soluble antigens that carry these types of carbohydrates.

In addition, this study provides the first comparison of the distribution of the MMR and DEC-205 on two human blood derived DC, Mo-LC and Mo-DC.

A panel of mAb that recognize several immunoglobulin-like transcript molecules (ILT) was also evaluated in this study. The mAb HP-F1 (CD85i) identified ILT2 [13], ZM3.8 (CD85j) recognized ILT3 [17], 42D1 (CD85f) identified ILT4 [16] and 7H5 (CD85a) recognized ILT5 [15]. One significant feature of the ILTs molecules is their capability to bind MHC class I molecules [13]. Furthermore, the molecules ILT2, ILT3, ILT4, and ILT5 have been considered to play a role as inhibitory receptors because they carry the immunoreceptor tyrosine-based inhibitory motif (ITIM) in the intracytoplasmic tail. Because of (i) the binding capability of ILT2-5 to MHC class I molecules [13,17], (ii) the presence of inhibitory motifs within the cytoplasmic tail of ILT2-5 [13,15-17] and (iii) the identification of ILT2-5, within diverse populations of dendritic cells [10,17], is likely that the ILT molecules identified on DC may not only perform a key role as the receptor for the "missing self [30] but also

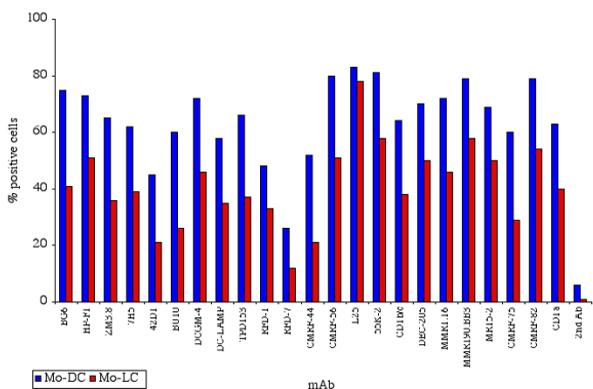


Figure 3
Percentage of positive cells detected by mAb against Mo-DC and LC-DC after 21 days of culture

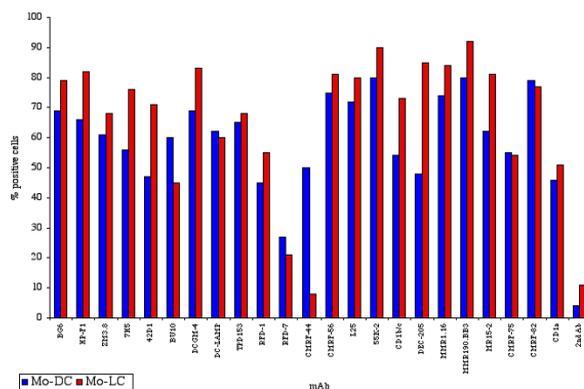


Figure 4
Percentage of positive cells detected by mAb against Mo-DC and LC-DC after 28 days of culture

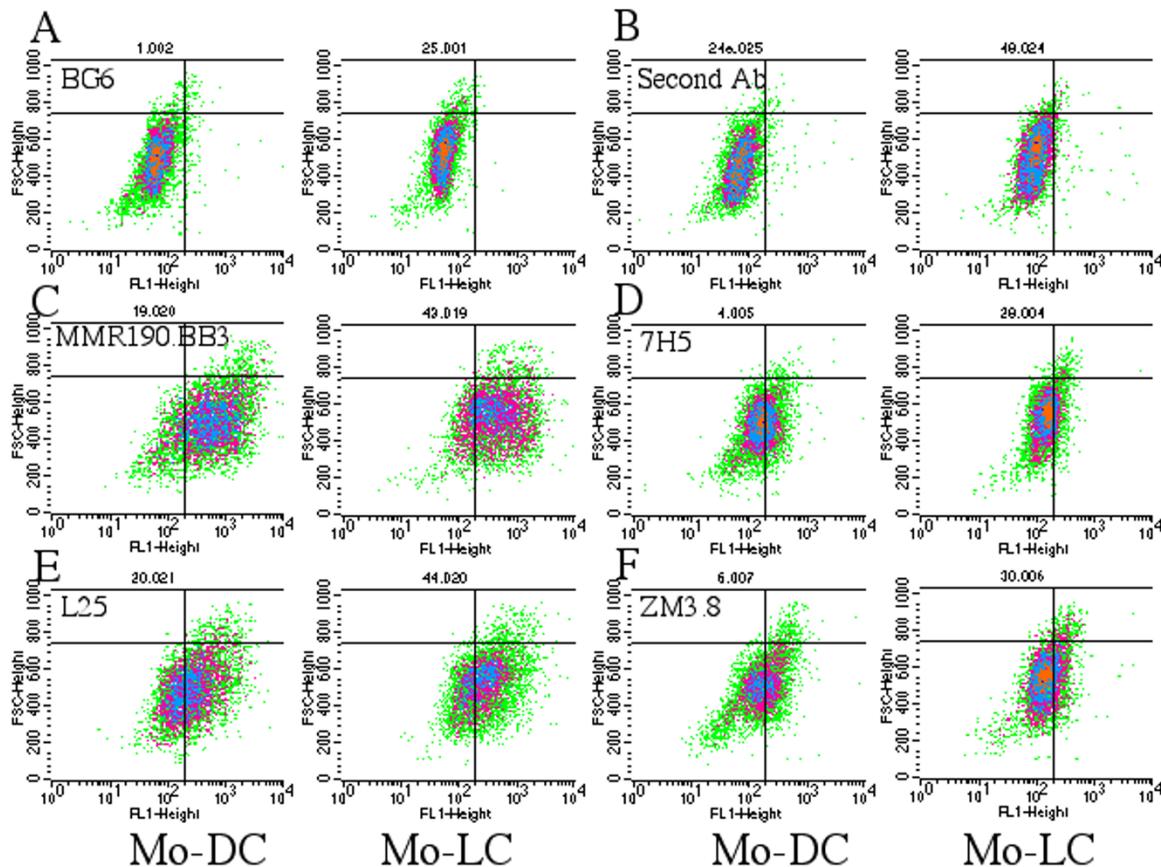


Figure 5

Density plots of Mo-DC and LC-DC reactivity to five mAb after 7 days of culture. Density plot representation of FSC and mAb reactivity at day seventh has been utilized to demonstrate the presence of significant variability within the Mo-DC and Mo-LC populations. Panel A displayed mAb-BG6 in comparison with the negative control that is showed in panel B-Second Ab. Panel C showed mAb-MMR190.BB3. Panel D displayed mAb-7H5. Panel E showed mAb-L25. Panel F displayed mAb-ZM3.8.

could contribute to control the initial steps of activation of the immune response; Thus, this would tune up the fine balance between the activation signals provided by the simultaneous cross-linking of MHC class I or II and co-stimulatory molecules during the interaction between DC with T cells and the inhibitory signals mediated through the whole variety of ILTs molecules carrying ITIM motifs. Further functional studies of the role of ILTs on DC will provide insight toward the understanding of these mechanisms and its role in the regulation of the DC function.

Conclusions

The immunophenotyping assays described in this report enabled us to determine in human DC: (i) the existence

of differences between Mo-DC and Mo-LC populations; (ii) the existence of subsets within the Mo-DC and Mo-LC populations; (iii) the kinetics of antigens expression at diverse intervals of time on DC; and (iv) specific markers for subpopulations of DC.

Material and methods

Monoclonal antibodies (mAb)

A panel of mAb with potential reactivity against DC was evaluated by setting up a flow cytometry approach. The panel of antibodies was kindly provided by Dr. D. Hart. Antibodies are listed in Table 21 {CD1a, CD1b/c, BG6, HP-F1 (CD85i), BU10, RFD-1, CMRF-44, 7H5 (CD85a), ZM3.8 (CD85j), 55K-2 (fascin), MMR1.16, MMR190.BB3 (CD206), L25, CMRF-56, RFD-7, MR15-2

Table 2: List of monoclonal antibodies

Sample	Panel number	Monoclonal antibody (CD [*])	Reference
1	70011	BG6	12
2	70194	HP-FI(CD85i)ILT2	13
3	70293	BU10	14
4	70345	7H5 (CD85a) ILT5	15
5	70346	42D1 (CD85f)ILT4	16
6	70358	ZM3.8 (CD85j) ILT3	17
7	70427	DCGM-4 (CD207) Langerin	18
8	70435	DC-LAMP (CD208)	19
9	70451	TPD153	20
10	70511	RFD-1	21
11	70512	RFD-7	22
12	70375	CMRF-44	7
13	70376	CMRF-56	23
14	70802	MR15-2 (CD206)	24
15	70808	DEC-205 (CD205)	6,12,25
16	70807	CD1b/c	31
17	70806	55K-2 fascin	26
18	70776	MMR1.16 (CD206)	27
19	70767	MMR190.BB3 (CD206?)	27
20	70772	L25	28
21	Positive Control 1	CMRF-75	12
22	Positive Control 2	CMRF-82	12
23	Positive Control 3	CD1a	31
24	Second Ab	2nd Ab	31
25	70875	AZN-D1 (CD209)	29
26	70876	AZN-D2 (CD209)	29
27	70874	MMRI-4 (CD205)	12,25

(CD^{*}): Hart DNJ., Clark GJ. MacDonald K., Kato M., Vuckovic S., Lopez A., Wykes M., Munster D.. 7th Leucocyte Differentiation antigen workshop, DC section summary. D. Mason, Editor, Leukocyte Typing VII, Oxford University Press. Oxford. In Press 2001 [12]

(CD205), DCGM-4 (CD207), TPD153, 42D1 (CD85f), DEC-205 (CD205), MMRI-4 (CD205), DC-LAMP (CD208), AZN-D1 (CD209), AZN-D2 (CD209), CMRF-75, CMRF-82, CD11c, CD80, CD86 and HLA-DR} [12–29,31]. The panel of antibodies that was utilized in the Dendritic cell section of the 7th workshop for HLDA is listed in table 2[12–29].

Cell culture

Adherent PBMC were obtained from a normal healthy donor and cultured in 25-cm² tissue culture flasks, with complete medium for DC culture {Complete Medium: RPMI 1640 with 25 mM Hepes buffer with L-glutamine (Gifco BRL, Basel Switzerland), supplemented with 10% heat-inactivated FBS, and 1 ml of Antibiotic/Antimycotic (Gifco BRL)} and supplemented with rhGM-CSF (250 ng/ml) +rhIL-4 (100 ng/ml) in order to generate Dermal DC like (Mo-DC). Adherent PBMC were also cultured in 25-cm² tissue culture flasks, with rhGM-CSF (250 ng/ml) +rhIL-4 (100 ng/ml) and rhTGF- β 1 (10 ng/ml) in order to generate DC like Langerhans cell (Mo-LC) [31]. Afterwards, the DC (Mo-DC and Mo-LC) cultures were

incubated at 37°C, 5% CO₂ for 7, 14, 21 and 28 days. Every week, half volume of the medium was removed and replaced with fresh medium without adding additional cytokines.

The Mo-DC and Mo-LC populations were harvested and evaluated at 7, 14, 21 and 28 days

Flow cytometry analysis

Cells (10⁵ cells/100 μ l) were incubated with 10 μ l of mAb on ice for 30'. Cells were washed, twice with wash buffer (Becton Dickinson, San Jose Ca), and stained with 10 μ l (1:100 antibody dilution) anti-mouse Ig FITC-labeled on ice for 30', washed once and fixed with Cellfix™ (Becton Dickinson, San Jose Ca). Thereafter, cells were acquired (10⁴ cells per sample) and analyzed on a FACSCalibur (Becton Dickinson, San Jose Ca). Cells were acquired and gated by FSC and SSC. The gated cells were analyzed by (i) histograms displaying the fluorescent reactivity collected in fluorescence 1 (green channel); (ii) Density plots displaying the fluorescent reactivity collected in fluorescence 1 (green channel) against the FSC [31]. Flow

cytometry measurement on non-stained or stained only with the second antibody (secondary antibody) were performed and used as control populations. Secondary antibody only stained cells at day 7 served to determine the markers and the quadrant borders. At least 99% of these cells were located in the lower left quadrant (negative). Three independent experiments were performed. Data were analyzed with CellQuest (Becton Dickinson, San Jose Ca). Statistical analysis (mean, standard deviation and graphics) was performed with Microsoft Excel.

Acknowledgement

This work was supported by grant 31-45903-95 of the Swiss National Science Foundation to Rafael Nunez and support of the Kanton of Zurich to RN and LF. We express our gratitude to Dr. D. Hart for providing us with the monoclonal antibodies and pre-published material. We thank D. Domingo (Memorial Sloan Kettering Cancer Center) for her review of the manuscript.

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