4. RESULTS

4.1 Characterization of antigen-specific TCC with different functional polarization

In order to study the role of the activated T lymphocytes on the differentiation and maturation of DCs, a panel of PPD and PJ specific TCC from normal donors was isolated. To obtain specific TCC with different functional polarization, it was set up primary culture of PBMC from a PPD^+ and PJ^+ healthy donor in the presence or absence of IL-4 (Maggi et al., 1992; Nisini et al., 1997). Positive T cell lines were cloned by limiting dilutions and clones screened for PPD and PJ specificity through the analysis of cells proliferation in the presence or absence of the antigen and autologous APCs (**Fig. 10**).

Then, selected TCC were characterized through a large phenotype analysis of CD8, CD4 (**Fig. 11A**) and three other surface molecules such as: CD40L, CD25 and CD62L before and after anti-CD3 stimulation (**Fig. 11B**).

Results show that all the $CD4^+$ TCC after anti-CD3 stimulation up regulate CD40L and CD25 and conversely down-regulate CD62L.

TCC were then tested for their capacity to secrete IFN-γ and IL-4, so they were stimulated in an antigen specific manner using autologous monocyte as APCs; the production of these two cytokines were then detected by intracellular cytokine staining and flow cytometric analysis (**Fig. 12**).

For a more complete functional characterization, the most relevant cytokines produced by the selected pool of Th1, Th2 and Th0 TCC was measured as mRNA transcript levels by RT PCR and as amount of proteins by ELISA.

Fig. 13 reports the cytokine release after TCC antigen specific PPD stimulation using monocytes as APCs.

To avoid the possible detection of cytokines released by APC, some of them were analyzed stimulating TCC with plastic bound anti-CD3 and the results are reported in **Tab. I**.

Th1 clones release IFN-γ, GM-CSF, TNF-α and IL-2 and increase their IL-6, IL-1β and IL-3 mRNA transcripts upon activation.

Conversely, the stimulation of Th2 clones is characterized by the release of IL-4 together with GM-CSF, IL-13 and IL-5.

Upon activation Th0 clones, in addition to the IL-4 and IFN-γ release, increase GM-CSF, TNF-α, IL-2 and IL-5 protein secretion and the mRNA induction of IL-1β, IL-3, with a remarkable expression of IL-6.

All the tested cytokines were induced specifically upon stimulation, since cytokines in

resting TCC were not detectable by ELISA and were detectable at extremely low levels by RT PCR (data not shown).

It is interesting to note that the amount of cytokines secreted by activated TCC, and in particular GM-CSF and IL-4, is of the same order of magnitude of that required to obtain monocytes differentiation *in vitro* using the recombinant cytokines.

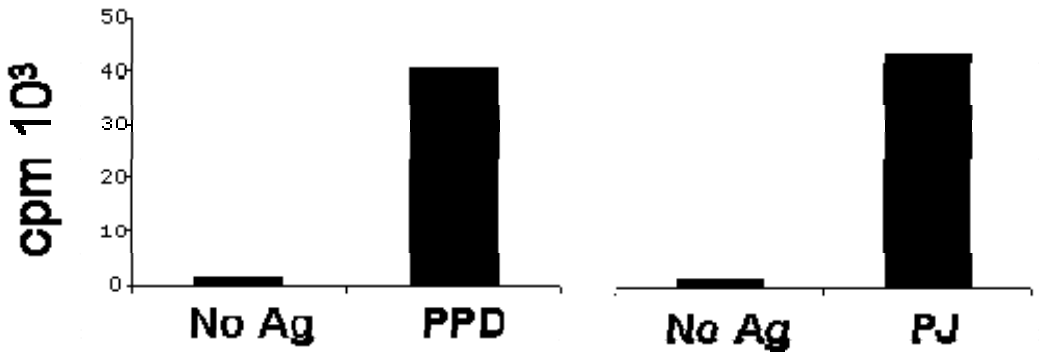


Fig 10. The isolated TCC are antigen specific

Positive lines derived from a PPD^+ and PJ^+ healthy donor were cloned and the clones were then screened for PPD and PJ specificity.
Specificity assays were performed in 96 well plates co-culting TCC with irradiated autologous PBMC pre-pulsed or not with antigens, in particular PPD (10 µg/ml) and PJ (50 µg/ml). Cell proliferation was determined by estimating incorporation of ^{3}H-tymidine into DNA.
Ag: antigen

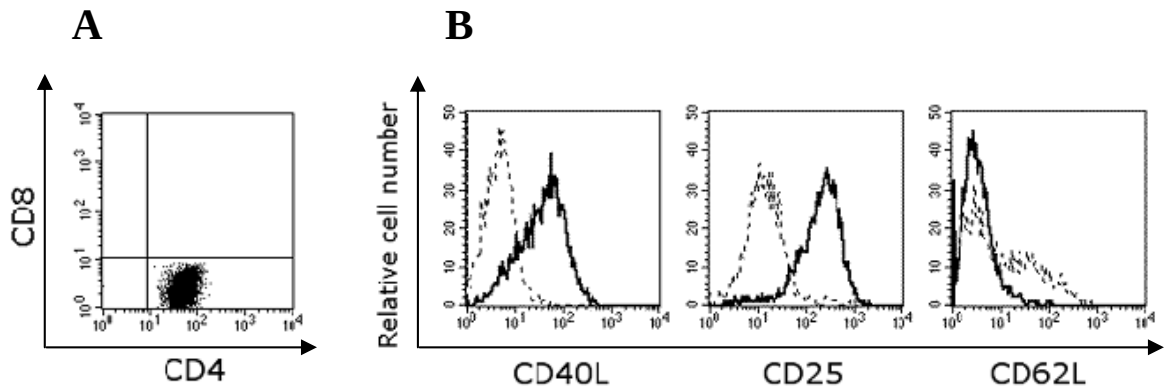


Fig 11. Phenotype of isolated antigen specific TCC

A) Double staining of antigen specific TCC for CD4 and CD8 expression were performed by immunofluorescence.
B) Histograms represents flow cytometric analysis of indicated surface molecules. Dotted histograms show staining of resting TCC, filled histograms represent staining of TCC after anti-CD3 stimulation.
Data are from one experiment representative of three independent experiments.
Similar results were obtained with four different clones per subset.

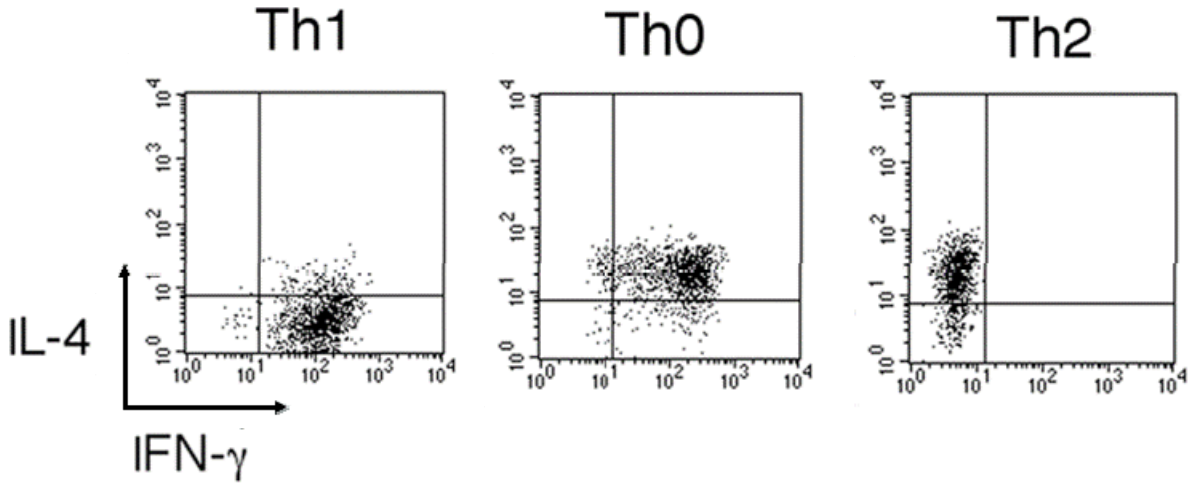


Fig 12. Functional polarization of TCC

PPD specific TCC were tested for their capacity to secrete IFN-γ and IL-4 by intracellular cytokine staining and flow cytometryc analysis.
Similar cytokine-patter was obtained with several clones per each subset.

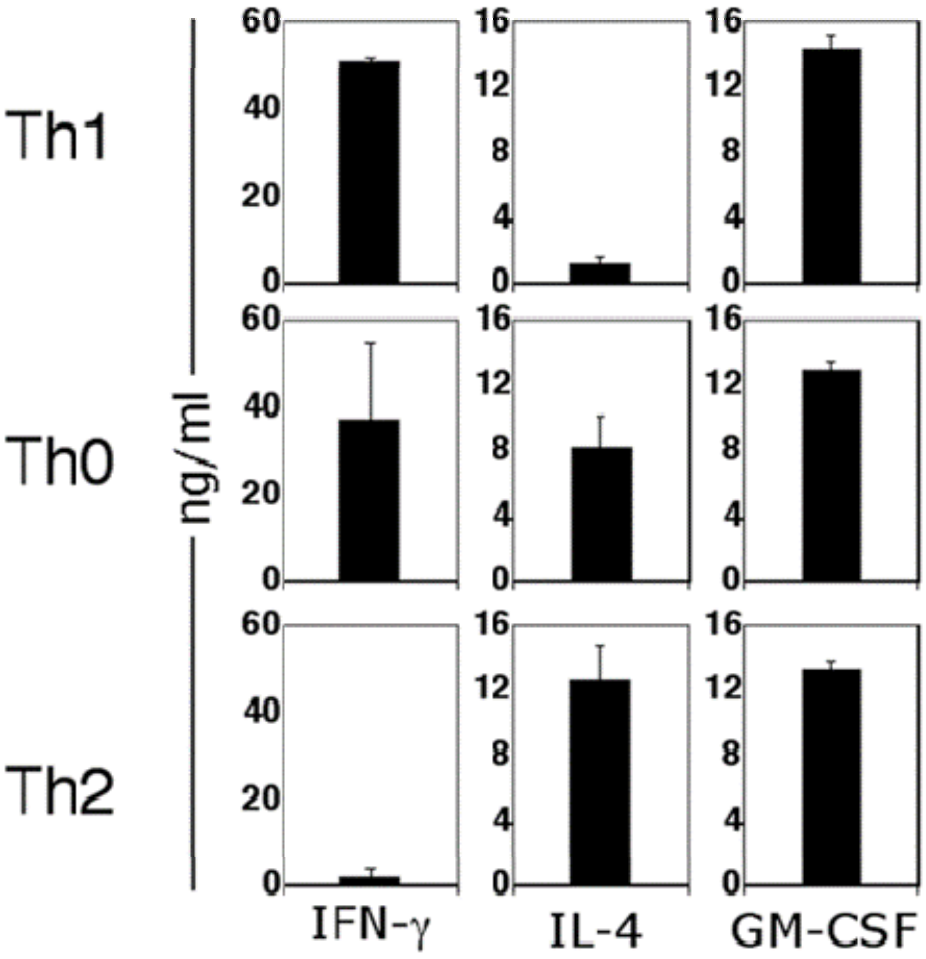


Fig 13. Characterization of cytokines released by TCC upon stimulation

Th1, Th0 and Th2 PPD-specific TCC and autologous isolated monocytes were co-cultured in the presence of PPD (10 μg/ml). After 3 days the supernatants were collected and the amounts of released IFN-γ, IL-4 and GM-CSF measured by ELISA.
Values indicate the mean of four different clones per each subset and are expressed as ng/ml ± SD.
The results are from one representative experiment out of ten. Similar results were obtained using at least four different clones per Th subset.

	Protein level[a]				
TCC	TNF-α (ng/ml)	IL-5 (ng/ml)	IL-13 (ng/ml)	IL-10 (ng/ml)	IL-2 (U/ml)[c]
Th1	19.1	4.5	1.1	0.5	361
Th0	12.7	19.8	n.d.	0	146
Th2	4.2	17.6	6.5	0	55

	mRNA transcript level[b]			
TCC	IL-6 (FI)	IL-1β (FI)	IL-15 (FI)	IL-3 (FI)
Th1	593	48	1	1150
Th0	1893	47	1	790
Th2	212	7	1	25

Tab I. Th1, Th0 and Th2 TCC synthesize different cytokines upon activation

[a] Amounts of secreted proteins after anti-CD3 stimulation of $5x10^5$/ml TCC were measured by ELISA and expressed as ng/ml.
[b] Levels of mRNA transcripts were expressed as fold induction (F.I.) calculated by ΔΔCt method using 18S mRNA level to normalize values and the mRNA level in non-activated TCC as a calibrator.
n.d. not determined.
[c] IL-2 released was measured using the CTLL2 proliferation assay and expressed as U/ml.

4.2 Specific T cell activation induces antigen-presenting monocytes to differentiate into DCs

To valuate if the specific T cell activation could induce antigen-presenting monocytes to differentiate into DCs, it was first set up a series of 6 days co-cultures of irradiated Th1 or Th2 TCC and autologous monocytes in the presence or absence of antigen (PPD) without the addition of any known differentiation factor.

After 6 days of culture, the phenotypic analysis of CD3-negative cells revealed that monocytes alone or co-cultured in the absence of PPD and irrespective of the Th1 or Th2 phenotype of the used TCC show a macrophage-like phenotype as revealed by microscopy and by their forward and side scatters (not shown) and CD14 expression (**Fig. 14**). On the contrary, in the presence of PPD, TCC induce the differentiation of PPD presenting monocytes into $CD14^{-ve}$ cells. These cells show a diverse CD1a expression

according to the Th1 or Th2 phenotype of the co-cultured clone. In particular, CD1a surface level in monocytes co-cultured with Th2 TCC is comparable to that of "reference" DCs generated in the presence of GM-CSF and IL-4 (DCk), while monocytes cultured with activated Th1 TCC exhibit a significantly reduced percentage of $CD1a^{+ve}$ cells (**Fig. 14**). These changes in monocyte phenotype are clearly dependent on the T cell activation, since PPD added to the culture is unable to interfere with the fate of monocyte cultured with or without GM-CSF and IL-4 (data not shown). These data indicate that, in addition to T cell activation, a consequence of antigen presentation is the differentiation of presenting monocytes.

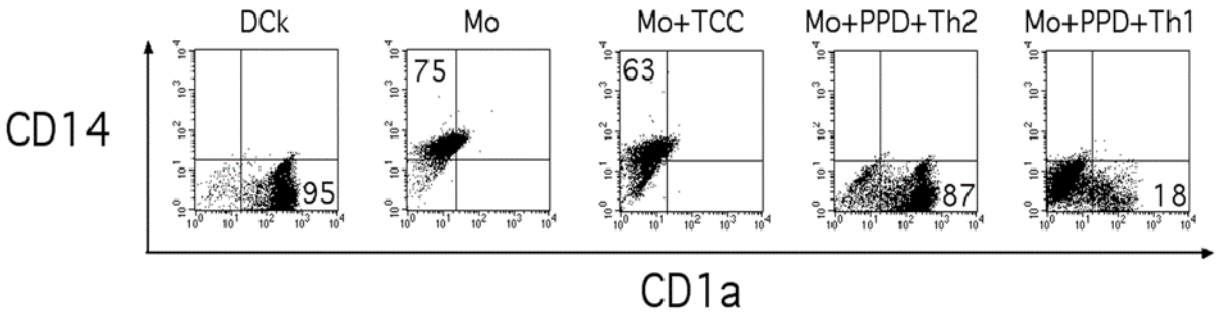


Fig 14. Expression of CD1a/CD14 on DC differentiated from monocytes co-cultured with antigen specific TCC

DCk were generated in a 6-days culture of monocytes with GM-CSF and IL-4. Autologous monocytes alone (Mo) or co-cultured with irradiated PPD-specific Th2 or Th1 TCC were incubated for 6 days in the absence (Mo+TCC) or the presence of PPD (Mo+PPD+Th2 and Mo+PPD+Th1 respectively).
Double staining analysis for CD1a and CD14 was performed on CD3 negative cells.
Numbers indicate the percentage of cells in each quadrant.

4.3 T cell-dependent monocytes differentiation into DCs does not require cell-to-cell contact

To test whether soluble factors released by activated TCC and/or their intimate interaction with monocytes are crucial to induce monocytes differentiation, it was also performed a series of experiments using a transwell culture system, where soluble cytokines released from cells in the upper chamber could diffuse to the lower chamber and interact with monocytes in the absence of cell-to-cell contact with TCC.

In the upper inserts, PPD-specific Th1, Th2 or Th0 TCC and autologous monocytes were placed with or without PPD or Parj1, while monocytes from the same or from a different donor were placed in the lower chamber.

Analysis of cells in the lower inserts after 6 days of culture showed that monocytes differentiate into cells with a level of CD1a molecule expression mirroring that of cells derived from monocytes co-cultured with Th1 and Th2 TCC in the presence of PPD. In the absence of antigen or in the presence of a non-related antigen, such as Parj1, monocytes do not differentiate into DCs and reveal a macrophage like phenotype. It is also showed that more than 70% of cells derived from monocytes sensing factors released by activated Th0 TCC are $CD1a^{+ve}$ (**Fig. 15**).

All together, data indicate that monocytes that have not processed and presented a given antigen, may undergo differentiation in a microenvironment in which other APC are responsible for T cell activation.

The DCs derived from monocytes sensing cytokines released from Th2, Th1 and Th0 are called respectively DCh2, DCh1 and DCh0.

4.4 Different TCC subpopulations induce monocytes to differentiate into DCs with distinct phenotypes

4.4.1 Morphologic characteristics of different DC subtypes

A morphologic analysis of DCh2, DCh1 and DCh0 in comparison to DCk has revealed that DCh2 as well as reference DCk were non-adherent cells while DCh1, and partially DCh0, were adherent, with long dendrites (**Fig. 16**) and had a morphology recalling that of DC recently activated by maturation stimuli such as LPS.

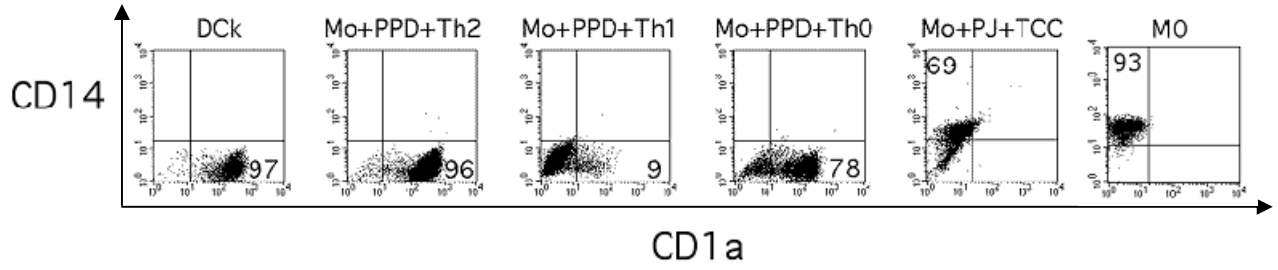


Fig 15. Soluble factors released by activated TCC induce monocytes differentiation

DCk were generated in a 6-days culture of monocytes with GM-CSF and IL-4. Monocytes were placed in the lower chamber of transwell plate whereas in the upper insert PPD-pulsed monocytes were co-cultured with autologous PPD-specific Th2 (Mo+PPD+Th2), Th1 (Mo+PPD+Th1) or Th0 (Mo+PPD+Th0) TCC. As a control of antigen specificity, PPD-specific Th2 or Th1 TCC were also co-cultured with Parj1-pulsed autologous monocytes (Mo+PJ+TCC) in the upper insert of transwell plate. Monocytes were also co-cultured with M-CSF (25 ng/ml) that allow their differentiation into cells with a macrophage-life phenotype (MO).
After 6 days, CD1a/CD14 surface expression of monocytes of the lower insert was checked.
Numbers indicate the percentage of cells in each quadrant.

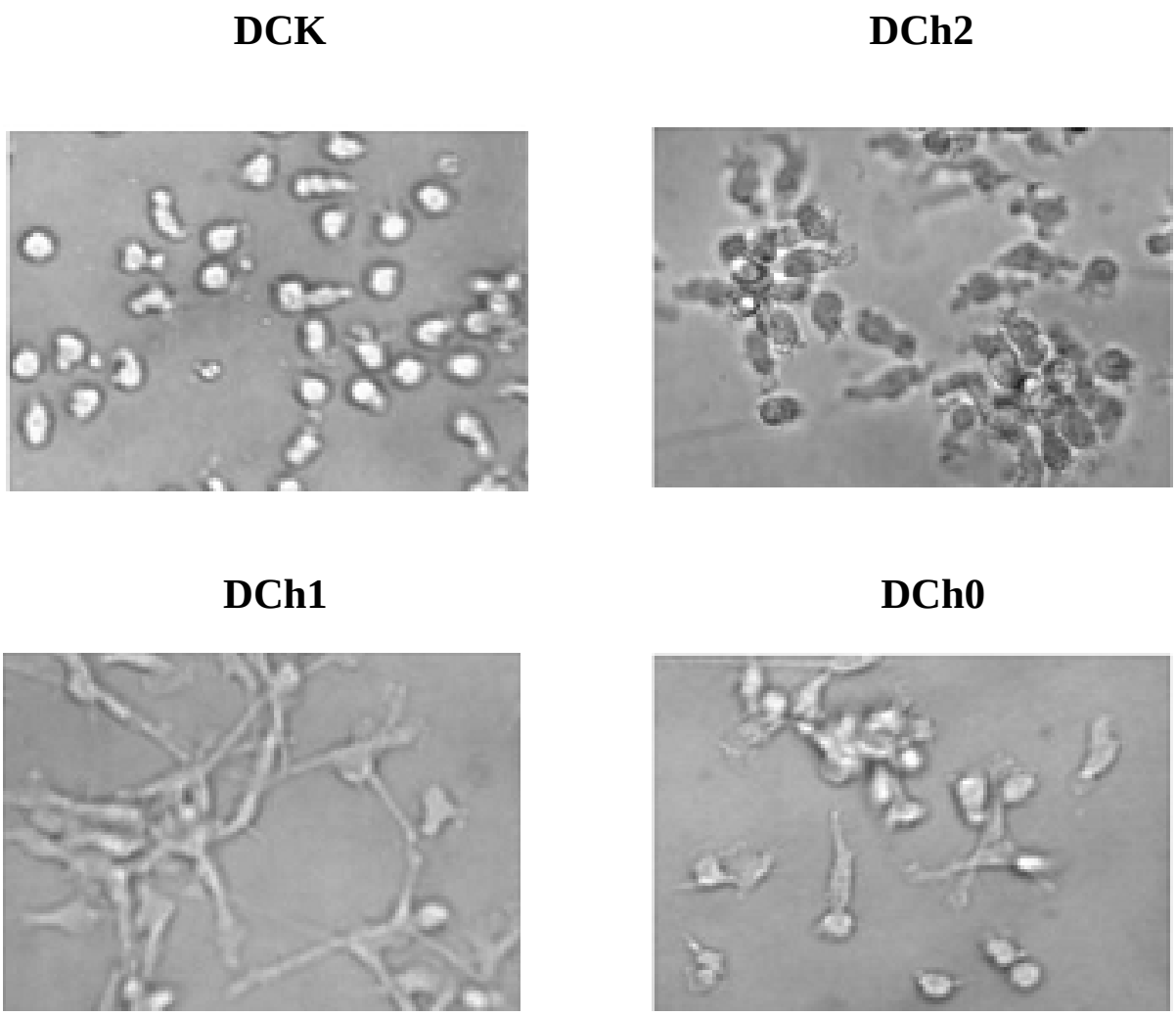


Fig 16. Morphological appearance of DCs differentiated upon TCC activation

Morphological analysis of the DC cultures on day 6 without LPS treatment. Cells were allowed to differentiate on round shaped coverslips seeded onto the bottom of 24 wells culture plastic plates and analyzed in phase contrast mode (400x).

4.4.2 Surface expression of group I CD1 molecules

The CD1 molecules comprise a small to moderate sized family of β2-microglobulin-associated, transmembrane glycoproteins, structurally related to the MHC-class I molecules (Dutronc and Porcelli, 2002).

In humans, the CD1 family has five members (CD1a, CD1b, CD1c, CD1d and CD1e) that are subdivided into three groups based on sequence homologies, sites of expression and function.

Thus, CD1a, CD1b and CD1c are typically classified as group 1 CD1 proteins, CD1d protein comprises group 2 and CD1e is classified as group 3 CD1.

Group 1 CD1 molecules have the unique capacity to bind microbial and endogenous lipid antigens and to present them to specific T cells (Angenieux et al., 2000; De Libero and Mori, 2006b).

Since the surface expression of group I CD1 molecules is considered one of the first distinct peculiarity of DCs, it has been conducted a flow cytometric analysis on the surface expression of these molecules on DCh2, DCh1 and DCh0.

Fig. 17 shows that DCh2 express high levels of CD1a, b and c, comparable to DCk; on the contrary DCh1 show much lower expression levels of CD1 molecules. The percentage of CD1 expression on the surface of DCh0 is between DCh2 and DCh1.

The expression of group I CD1 molecules is considered a distinct but not unique characteristic of monocytes derived DCs.

Moreover, CD1 expression is not a DCs marker, since $CD1^{-ve}$ DCs have been described (Chang et al., 2000; Gogolak et al., 2007).

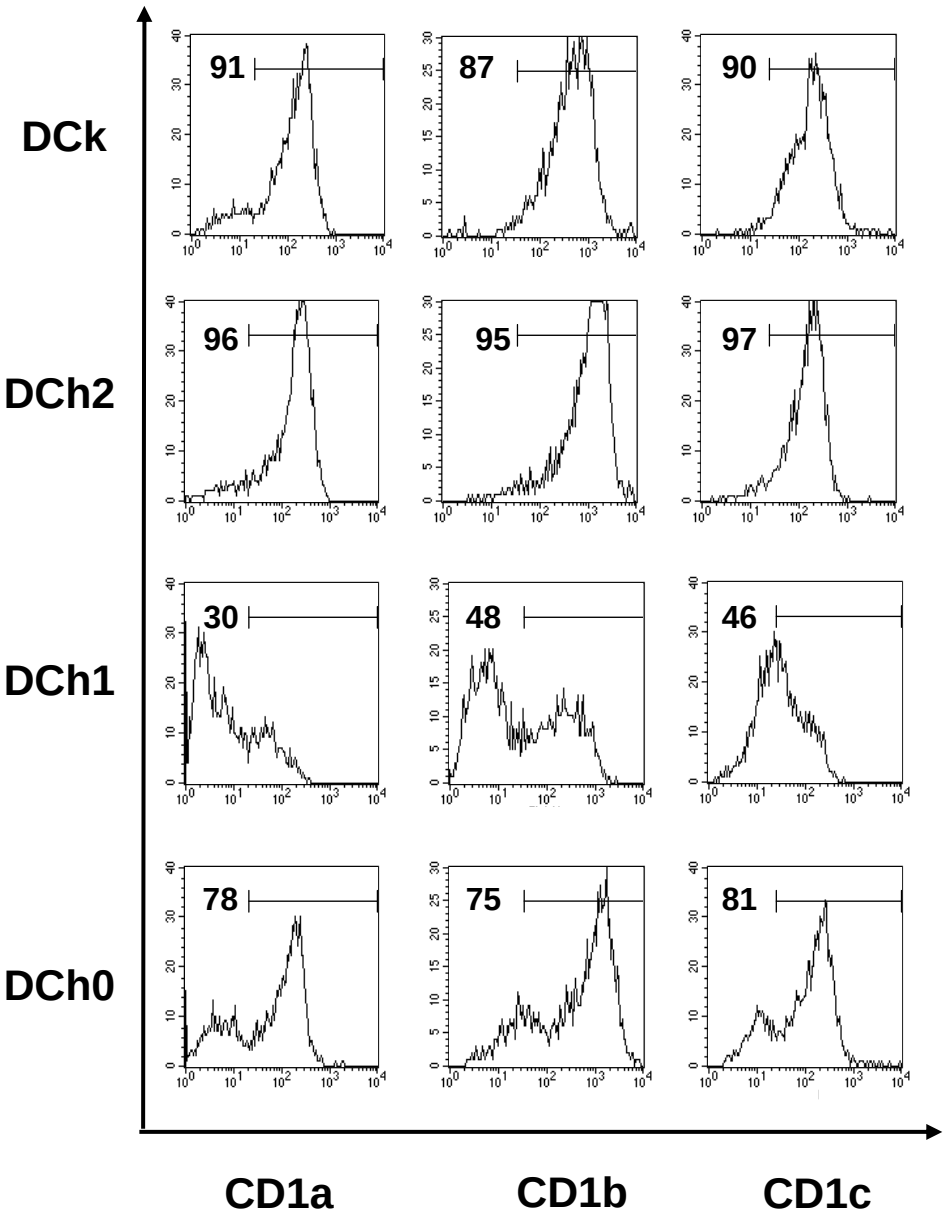


Fig 17. CD1 molecules expression by DCs differentiated from monocytes upon activation of different Th subpopulations

Monocytes were cultured for 6 days with GM-CSF and IL-4 (DCk) or in the lower chamber of a transwell device in which Th2, Th1 or Th0 TCC were co-cultured with autologous antigen pulsed monocytes in the upper chamber. DC derived from monocytes sensing cytokine released from Th2, Th1 or Th0 TCC were defined DCh2, DCh1 and DCh0 respectively.
The percentage of positive cells is reported in the relative histogram.

4.4.3 DC-SIGN expression

It has been largely demonstrated in literature that during DCs differentiation, the expression of DC-SIGN, a type II transmembrane protein that belongs to the C-type lectin family, expressed by monocyte-derived DCs, as well as by tissue DCs of virtually all body compartments, is primarily induced by IL-4, while TGF-β, IFN-α, or dexamethasone prevent its IL-4-dependent induction on monocytes (Geijtenbeek et al., 2000; Relloso et al., 2002).

Thus it was studied its expression on the surface of DCs (**Fig. 18**) and the results demonstrated that, in agreement with the direct dependency of DC-SIGN expression from the presence of IL-4 and the absence of IFN-γ in the culture, in comparison to DCk, a reduced percentage of DCh1 are DC-SIGN^{+ve}, and the majority of DC-SIGN^{+ve} cells are CD1a^{-ve} (Fig. 2C).

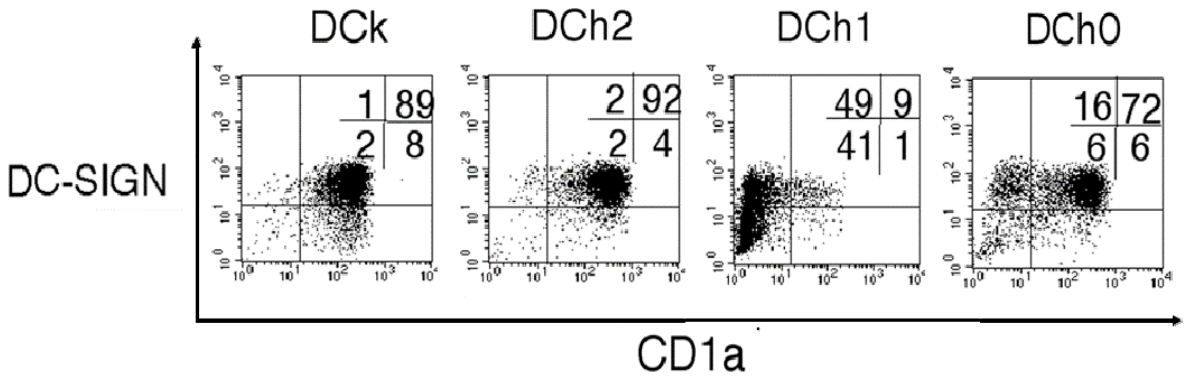


Fig 18. DC-SIGN surface expression in function of TCC

Surface expression of CD1a and DC-SIGN was analyzed by flow cytometry on DCk, DCh2, DCh1 and DCh0. Numbers indicate the percent of cells in corresponding quadrant.
The results are from one representative experiment out of three independent experiments.

4.4.4 Larger phenotypic characterization of DCs derived from monocytes cultured with activated Th2, Th1 or Th0 PPD specific TCC

To clearly establish the nature of cells derived from monocytes cultured with activated TCC, it was performed a larger phenotypic characterization of monocytes cultured with activated Th2, Th1 or Th0 PPD specific TCC in comparison to DCk. Monocytes cultured with Th2 TCC differentiated into DCs (DCh2) not distinguishable from DCk (**Fig 19**).

In fact, both these DC populations homogeneously express high levels of presenting molecules and MHC class I and class II (DR) molecules and are equally able to undergo maturation following LPS-treatment as assessed by the up-regulation of activation markers (CD83, CD80, CD86, CD40, MHC class I and II).

Monocytes cultured with activated Th1 and Th0 TCC differentiate into DCs (DCh1 and DCh0, respectively) with phenotypic characteristics different from DCk. In fact the level of maturation markers (CD83, CD86, DR) is higher than that of immature DCk. Interestingly, DCh1 and DCh0 do not markedly modify their phenotype after LPS treatment, with the exception of CD86, which is up regulated in DCh0.

All the DC subsets are $CXCR4^{-ve}$ and do not up-regulate this receptor after LPS (data not shown).

On the other hand, DCk and DCh2 turn $CCR7^{+ve}$ after LPS stimulation, while DCh1 and DCh0 were $CCR7^{+ve}$ even if the expression is lower than in mature DCk and do not change after LPS treatment. All the DC subsets are $CCR5^{+ve}$, but while DCh2 and DCk turn $CCR5^{-ve}$, DCh0 and DCh1 only reduce the expression of this receptor after LPS treatment.

4.4.5 CD1a-reduced expression on DCh1 and their semi-mature phenotype is related to IL-4 absence and/or IFN-γ secretion

In order to get some light on the reasons underlying the reduced CD1a expression on DCh1 and their quite mature phenotype, the attention was focalized on the role of IL-4 and IFN-γ. Since the absence of IL-4 into activated Th1 supernatant could be responsible for the lack of CD1 expression, monocytes were cultured for 6 days with Th1-supernatans in the presence of rhIL-4 added from the beginning of the culture. The addition of this cytokine is sufficient to cause a CD1a surface expression on derived DCs at level of DCk (**Fig. 20A**). Addition of IL-4, however, do not reduce the mature phenotype of DCh1 (**Fig. 20B**). On the other hand, since the presence of IFN-γ could inhibit CD1a expression monocytes were

cultured with Th2 supernatant in the presence of rhIFN-γ supplemented at day 0 of culture. **Fig. 20C** clearly shows the inhibitory effect of this cytokine in surface expression of CD1 molecules. Moreover, DCh2 differentiated in the presence of IFN-γ show a more mature phenotype than non-treated cells similar to DCh1 (**Fig. 20D**).

To confirm the contribution of IFN-γ in causing the reduced CD1 expression and the mature phenotype of DCh1, monocytes were cultured in the presence of GM-CSF and rhIFN-γ. The differentiated cells show a DCh1 like phenotype with low surface level of CD1a molecules and up-regulation of maturation markers (**Fig. 21**).

In summary this set of experiments suggests that the IL-4 absence and the IFN-γ presence drive the monocyte differentiation into DC with a reduced CD1a expression and a mature phenotype typical of DCh1.

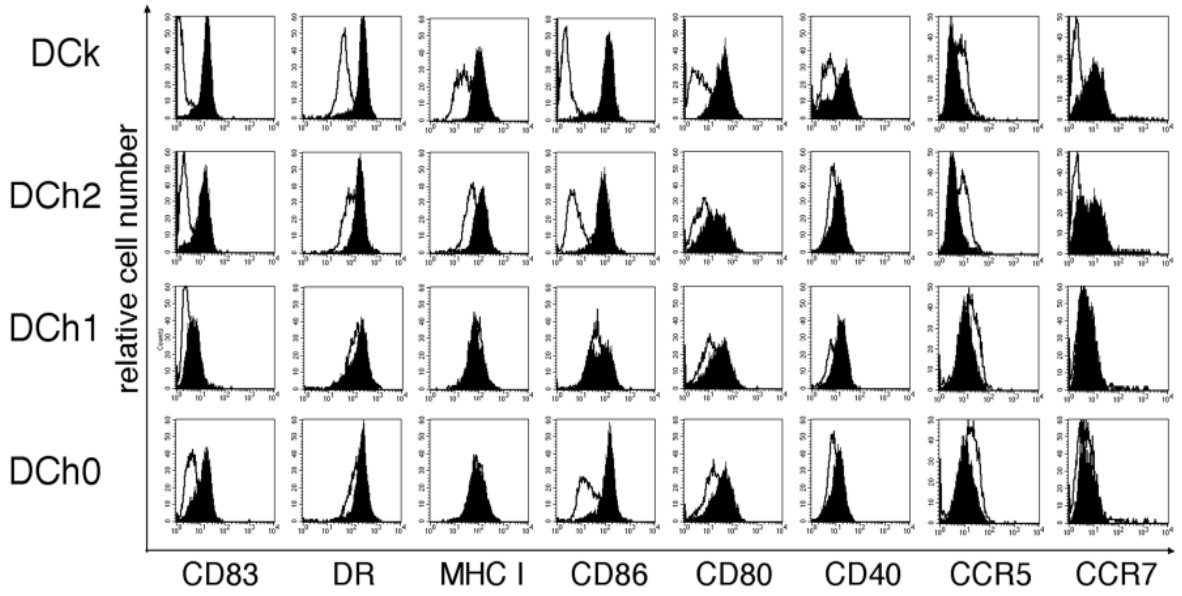


Fig 19. Phenotypical characterization of monocytes differentiated into DC with distinct phenotypes upon activation of different Th subpopulations

Flow cytometric analysis of indicated surface molecules with or without LPS stimulation.
The open histograms show staining of non-LPS stimulated cells; filled histograms show staining of the corresponding LPS-treated cells.

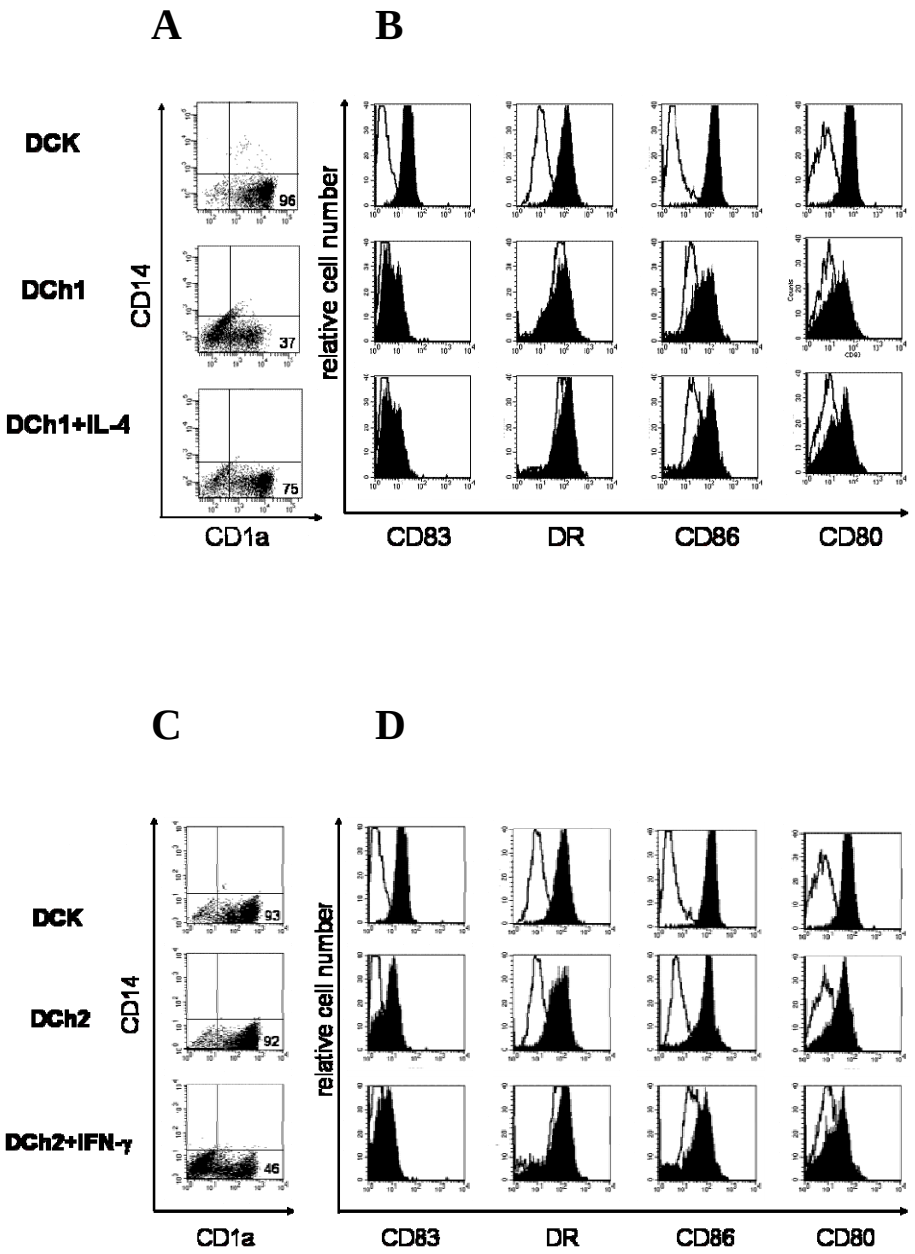


Fig 20. rhIL-4 and rhIFN-γ added to the cultures revert the effects of cytokines released by TCC on DCs

Monocytes were cultured for 6 days with IL-4/ GM-CSF (DCk), Th1-supernatans (DCh1) in the absence or presence of rhIL-4 (DCh1 and DCh1+IL-4 respectively) **(A-B)**, or Th2-supernatant (DCh2) in the absence or presence of rhIFN-γ (DCh2 and DCh2+ IFN-γ respectively) **(C-D)** added from the beginning of the culture. In **A** and **C** are represented double staining analysis for CD1a and CD14. Numbers indicate the percentage of cells in the corresponding quadrant.

B and **D** show flow cytometric analysis of indicated surface molecules with or without LPS stimulation. The open histograms show staining of non-LPS stimulated cells; filled histograms show staining of the corresponding LPS-treated cells.

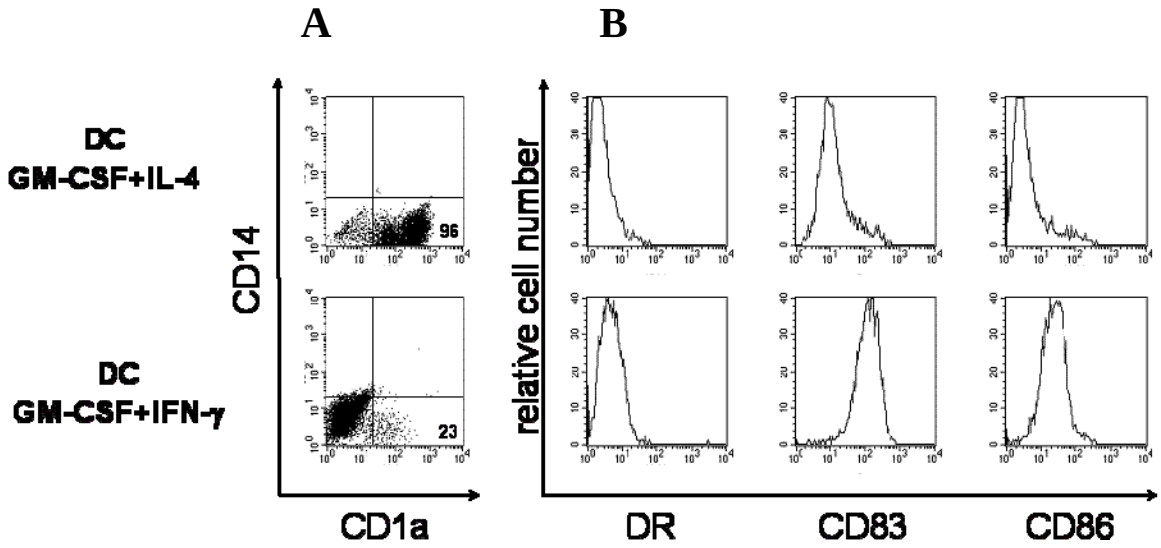


Fig 21. rhIFN-γ contribute to generate DCs showing a DCh1 like phenotype with low surface level of CD1a molecules

Monocytes were cultured in the presence of GM-CSF and rhIFN-γ in order to confirm the contribution of IFN-γ in causing the reduced CD1 expression and the mature phenotype of DCh1.
Numbers indicate the percentage of cells in each quadrant. Histograms show the maturation markers staining of non-LPS stimulated cells.
In A is represented a double staining analysis for CD1a and CD14. Numbers indicate the percentage of cells in the correspondent quadrant
B shows flow cytometric analysis of indicated surface molecules in the presence of GM-CSF and rhIL-4 or rhIFN-γ added from the beginning of the culture.

4.4.6 Freshly isolated T lymphocytes ex-vivo induce monocytes to differentiate into DCs

To test whether the ability to induce monocytes differentiation into DCs was an *in vitro* acquired function of cultured and IL-2 expanded TCC or a general function of T lymphocytes upon activation, an *ex-vivo* test was set up in order to analyze the phenotype of monocyte after 6-days culture in the presence of superantigen-activated freshly isolated autologous $CD4^+$ lymphocytes. After a 6 days of co-culture with SEA activated, but not with resting T lymphocytes, monocytes differentiated into $CD14^{-ve}$ and $CD1a^{+/-ve}$ DC (**Fig. 22**) with phenotypic characteristic similar to DCh1 (data not shown).
To note that SEA added at the same concentration did not induce DCs differentiation.

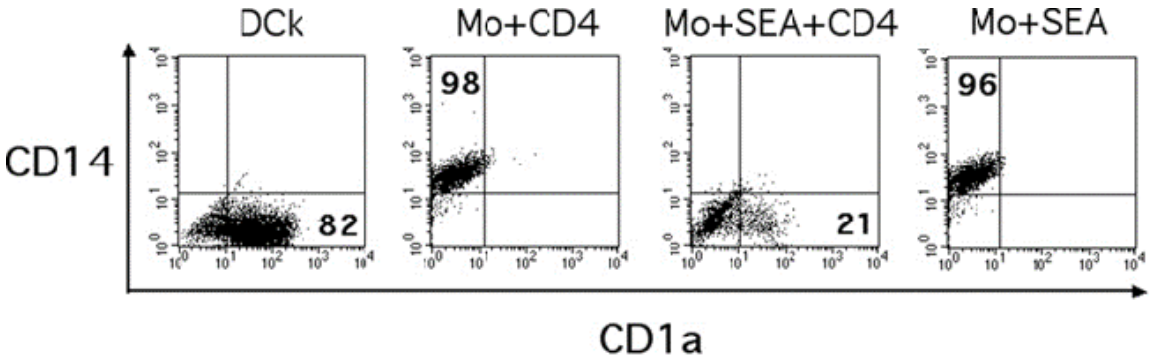


Fig 22. *Ex-vivo* T lymphocytes stimulation by a superantigen induces monocytes to differentiate into DCs

Freshly isolated $CD4^+$ T lymphocytes and autologous monocytes were co-cultured in the presence (Mo+SEA+CD4) or absence (Mo+CD4) of SEA at 0.1 μg/ml. After a 6 days culture, CD1a/CD14 expression was analyzed in the $CD3^{-ve}$ population. DCk were generated in a 6-days culture of monocytes with GM-CSF and IL-4.
Numbers indicate the percentage of cells in the relative quadrant.
The results are from one representative experiment out of three independent experiments performed with different healthy donors.

4.5 DCs differentiated upon T cell activation show a differential cytokine secretion pattern

To measure the cytokines released by DCs, cultures were washed after 5 days, cells counted and adjusted to 3 x 10^5 cell/ml and cultured in the presence or absence of 0.2 μg/ml LPS for an additional 18 h before supernatants collection. DCh1 and DCh0 produce low amounts of IL-12p70 even after LPS stimulation and their secretion is statistically different from that of DCk ($p<0.001$). On the other hand, LPS-matured DCh2 release amount of IL-12p70 not statistically different from that released by mature DCk (**Fig. 23**). Notably, DCh1 are characterized by their capacity to spontaneously release amounts of IL-10 significantly ($p<0.05$) higher than LPS stimulated DCk and the maturation stimulus do not significantly increase its release. DCh0 secreted IL-10 after LPS treatment at level significantly ($p<0.05$) higher than mature DCk. RT PCR analysis also show that IL-1β and IL-6 mRNA transcripts are detected upon activation in both DCh1 and DCh0, which show the highest expression of TNF-α transcripts among the different DC populations (data not shown).

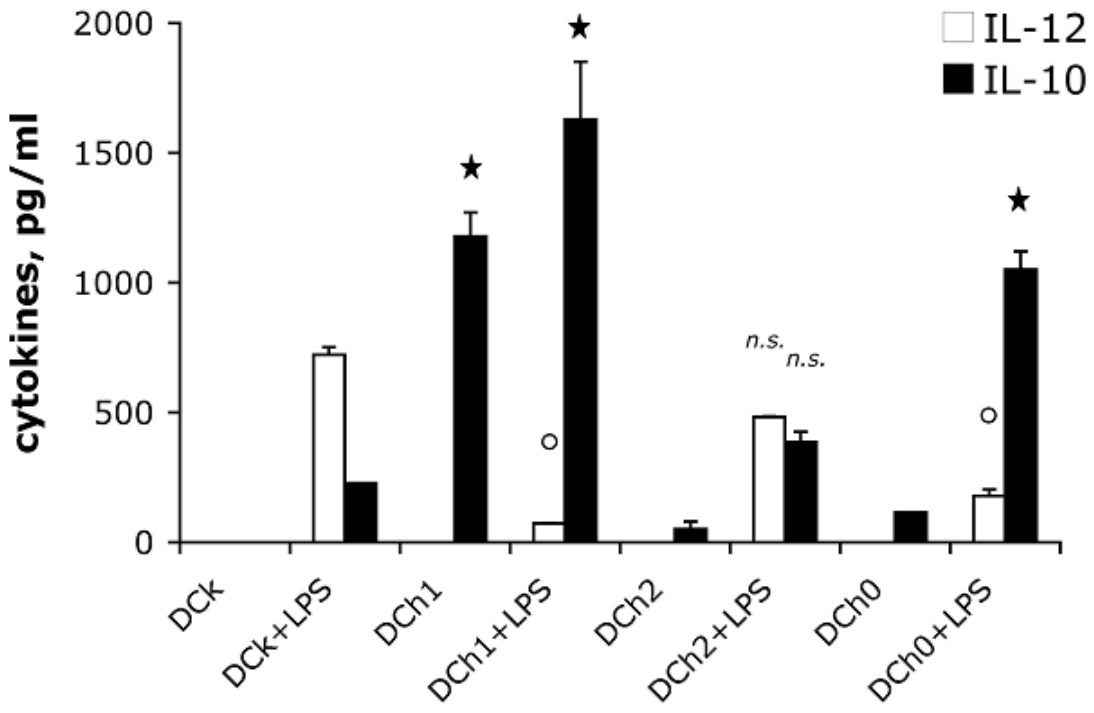


Fig 23. Cytokine secretion pattern of DC populations differentiated upon T cell activation

At day 5th of culture the DCk, DCh2, DCh1 and DCh0 cells were incubated in the presence or absence of 0.2 µg/ml LPS for an additional 18 h. Supernatants were collected at the end of the culture and examined for IL-12p70 and IL-10 contents by ELISA. Values indicate the mean of three independent experiments and are expressed as pg/ml ± SD.

★ indicates $p<0.05$ *vs* IL-10 released by DCk + LPS; ○ indicates $p<0.001$ *vs* IL-12 released by DCk + LPS; *n.s.* indicates not significant differences *vs* DCk + LPS.

4.6 DCs induced by activated T lymphocytes show different degree of bacterial phagocytosis and soluble antigen uptake

It is largely known that DCs circulating in the peripheral tissues in an "immature" state have the important role to uptake antigens through endocytic or phagocytic receptors and start the adaptive immune response (Banchereau et al., 2000; Banchereau and Steinman, 1998).

The capacity of DCs derived from monocyte upon TCC activation to phagocytose Gfp-rBCG is reported in **Fig. 24**. The percentage of fluorescent-bacteria associated cells is comparable between DCh2 and reference DCk populations (DCh2=20% and DCk=23%). As expected, these percentages are reduced upon LPS-stimulation. On the contrary, a lower

percentage of DCh1 (10%) and DCh0 (13%) bind Gfp-rBCG after 1 h of incubation, as compared to reference cells, consistent with the partial maturation state of these DCs (**Fig. 24A**). Interestingly, however, the low internalization capacity of these cells was further reduced after LPS stimulation, suggesting a sensitivity to this maturation stimulus that is not observed in terms of surface molecules expression (**Fig. 24A and B**).

Similar results are obtained also evaluating the capacity of FITC-albumin endocytosis (**Fig. 25A and B**) as a measure of soluble antigen uptake.

A flow cytometric analysis was conducted on DCs and it has been detected that after 1 hour of culture of DC in the presence of FITC-albumin at 37°C, DCk and DCh2 show a reduction of endocytosis capability after LPS stimulation, on the contrary DCh1 and DCh0 do not show a decrease of endocytosis after LPS treatment probably due to the fact that also in the immature state these cells don't have an high endocytosis capacity.

4.7 DCh1 and DCh0 have a reduced capacity to prime naïve $CD4^+$ T lymphocytes

The antigen presenting capability of the diverse monocyte-derived DC populations was evaluated in a MLR using a sorted population of allogeneic cord blood $CD4^+$ T lymphocytes as responder cells. DCh1, DCh2 and DCh0 generated from monocytes in the lower chamber of a transwell device were used in order to obtain DCs non-contaminated by irradiated T cells. All the non-LPS treated DCs are unable to stimulate an efficient proliferation of naïve T cells, which, on the other hand, proliferate extensively when stimulated by LPS-matured DCh2 and DCk. Interestingly, LPS matured DCh1 and DCh0 induce a T-cell proliferation that is constantly of lower magnitude than the proliferation induced by mature DCk (**Fig. 26**).

Analysis of intracellular cytokine production by naïve $CD4^+$ T cells expanded in MLR show that mature DCh2 induce the expansion of a number of IFN-γ and IL-2 producing T cells comparable to DCk (**Fig. 27A-B**). To note, DCh2 prime a reduced number of cells secreting IL-4, indicating that they are capable of inducing a more marked Th1 response than DCk. Inversely, among the T cells polarized by mature DCh1 and DCh0, a statistically significant reduced percentage of IFN-γ and IL-2 secreting cells and a higher proportion of cells producing IL-10 in T expanded by DCh0 are observed. ELISA measurement also confirmed that IL-10 was released by T cells stimulated by DCh0 in amounts statistically higher ($p<0.01$) than those released by T cells primed by other APC (**Fig. 27B**).

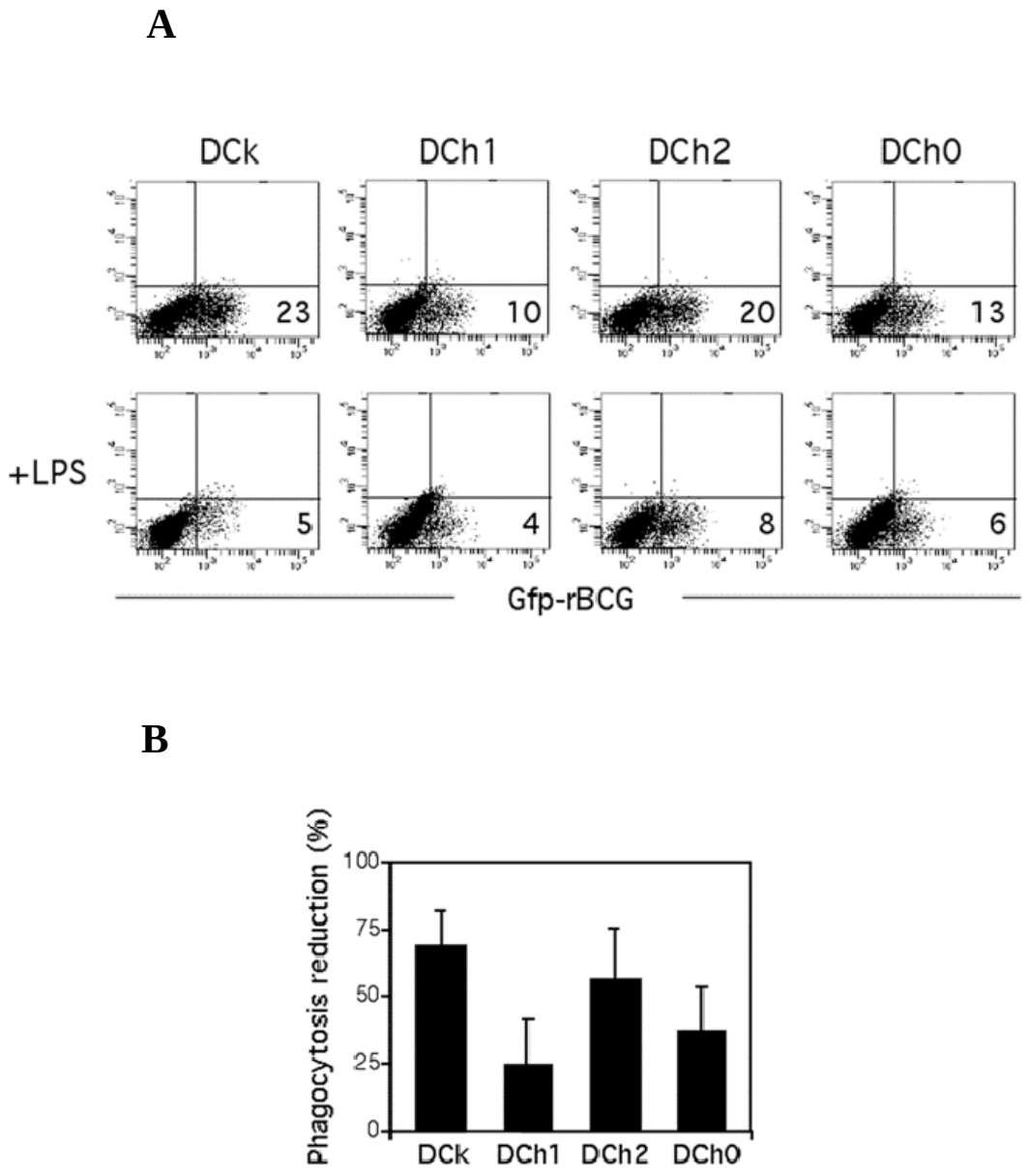
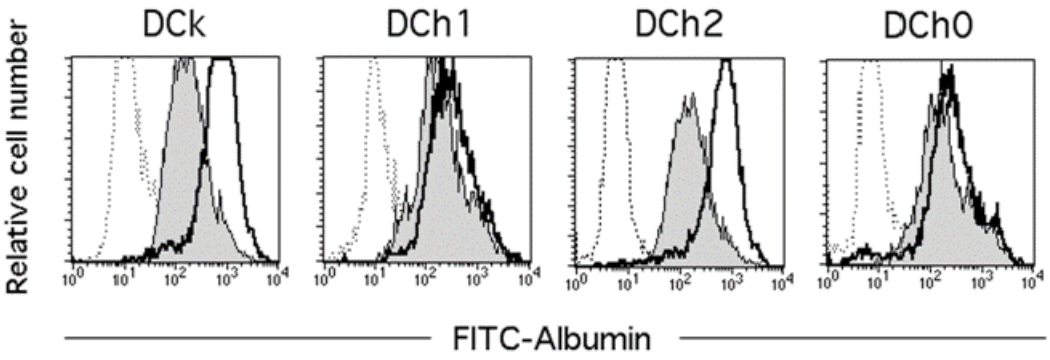


Fig 24. Phagocytosis of DC populations differentiated upon T cell activation

At day 5th of culture the DCk, DCh2, DCh1 and DCh0 cells were incubated in the presence or absence of 0.2 µg/ml LPS for an additional 18 h.
A) At the end of the cell culture DCs were incubated 1 h at 37°C with Gfp-rBCG at a multiplicity of infection cell:BCG=1:6 in CM supplemented with 10% FCS and then washed by low-speed centrifugation (100*g*). The percentage of cells that bound Gfp-rBCG was evaluated by flow cytometry and the number indicated in the quadrant. The results are from one representative experiment out of four independent experiments.
B) Reduction of phagocytic activity after LPS-induced maturation. Results are expressed as reductions in the percentages of Gfp-rBCG phagocytosis upon LPS-stimulation $\pm$ standard deviations of four independent experiments

A



B

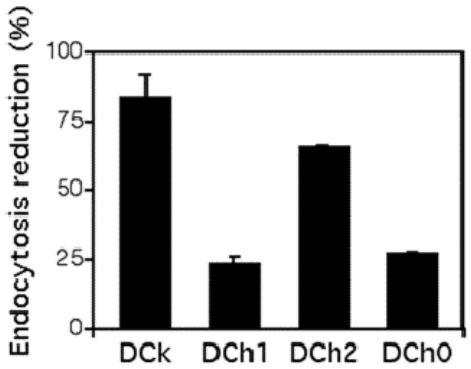


Fig 25. Endocytosis of DC populations differentiated upon T cell activation

At day 5th of culture the DCk, DCh2, DCh1 and DCh0 cells were incubated in the presence or absence of 0.2 µg/ml LPS for an additional 18 h.
A) Analyses by flow cytometry of FITC-albumin uptake after 1 hour at 0°C (negative control; dotted histograms) or 37°C of immature (empty black histograms) and LPS-stimulated (filled grey histograms) DC.
B) Reduction of endocytosis activity after LPS-induced maturation. Results are expressed as reductions in the mean fluorescent intensity upon LPS-stimulation $\pm$ standard deviations of four independent experiments

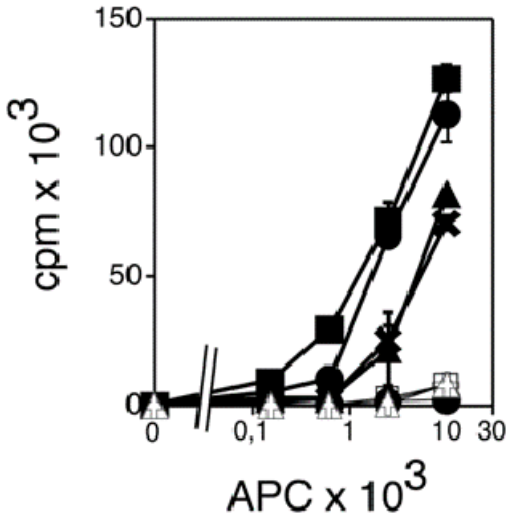


Fig 26. Mature DCh1 and DCh0 induced a T-cell proliferation lower than that induced by mature DCk

DCk (squares), DCh2 (circles), DCh1 (triangles) and DCh0 (crosses) at day 5th of culture were incubated in the absence (open symbols) or presence of 0.2 μg/ml LPS (filled symbols) for an additional 18 h and used as APC at different cell numbers to stimulate 3 x 10^4 cord blood-purified $CD4^+$ T cells.
The T cell proliferative response was measured after 6 days by ^{3}H-thymidine incorporation and results expressed as mean cpm of triplicate wells.
One experiment representative of six is shown.

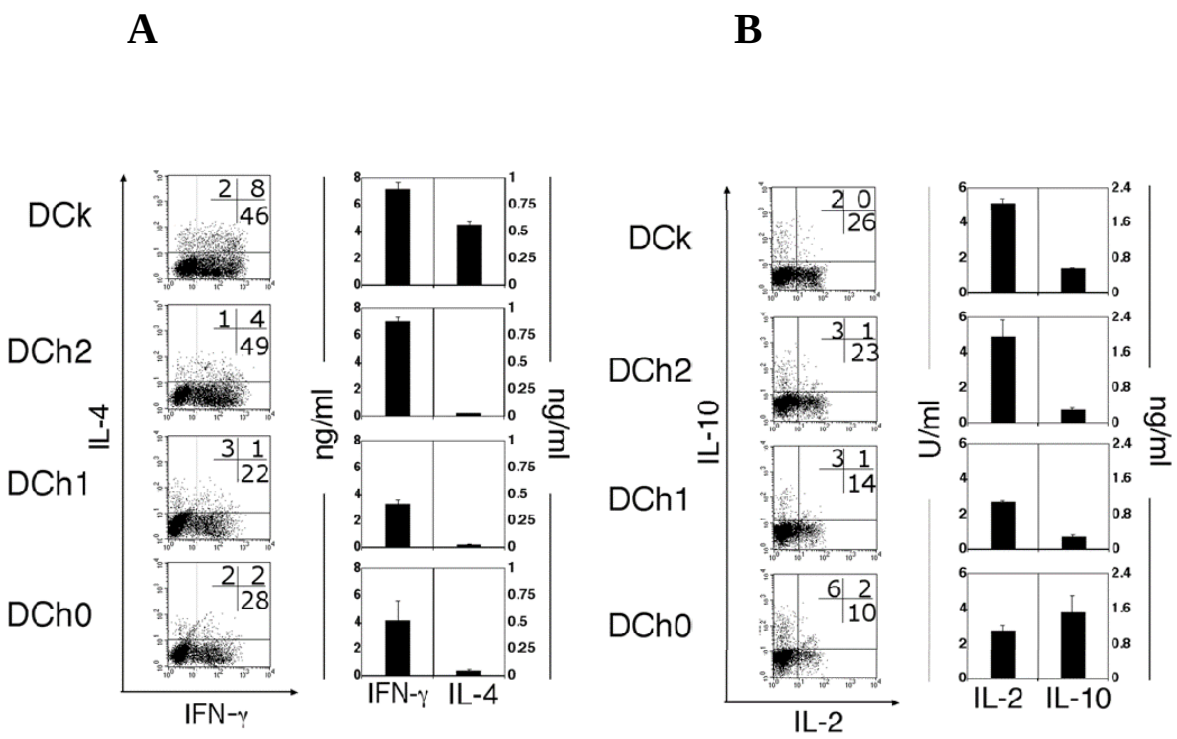


Fig 27. Analysis of intracellular cytokine production by naïve CD4$^+$ T cells

Cytokine production by naïve CD4$^+$ T cells after co-culture with allogeneic LPS treated DCk, DCh2, DCh1 and DCh0. Dot plots represent the flow cytometric analysis of intracellular cytokine accumulation and numbers indicate the percentage of cells in the corresponding quadrant. Histograms represent cytokines released in supernatants as measured by ELISA (ng/ml $\pm$ SD) with the exception of IL-2, which was measured by a biological assay using the cell line CTLL-2 and expressed as U/ml $\pm$ SD

4.8 DCh1 and DCh0 have a reduced capacity to activate antigen- specific memory T-cell response

To better characterize the functional capability of the different monocytes-derived DCs, it was evaluated their capacity to present a soluble antigen to TCC, as a model of secondary immune response.

In details, DCs were compared for their capacity to present PPD to a specific $CD4^+$ TCC and a lipid antigen to a CD1b restricted TCC.

The ability of the different DCs to present PPD and PIM_2 to specific MHC class II and CD1 restricted TCC respectively was analyzed.

As reported in **Fig. 28A**, the efficiency of presentation, measured as the PPD concentration required to give 50% of maximum TCC proliferative response, varies with APC. The antigen presentation capacity of DCh2 is comparable to that of DCk, while DCh1 and DCh0 showed a reduced efficiency.

Interestingly, DCh2 are even more efficient than DCk to present a lipidic antigen to a specific CD1b-restricted TCC (**Fig. 28B**). On the contrary, DCh1 and DCh0 show a reduced capacity to activate the specific TCC, both in term of maximum response and amount of antigen required, that can be only partially attributed to the reduced expression of surface CD1b-molecules.

Since DCh1 and DCh0 show high level of presenting and co-stimulating molecules compared to control DC, their reduced antigen presentation capacity can be explained by the different set of released cytokines and by their reduced up take capability.

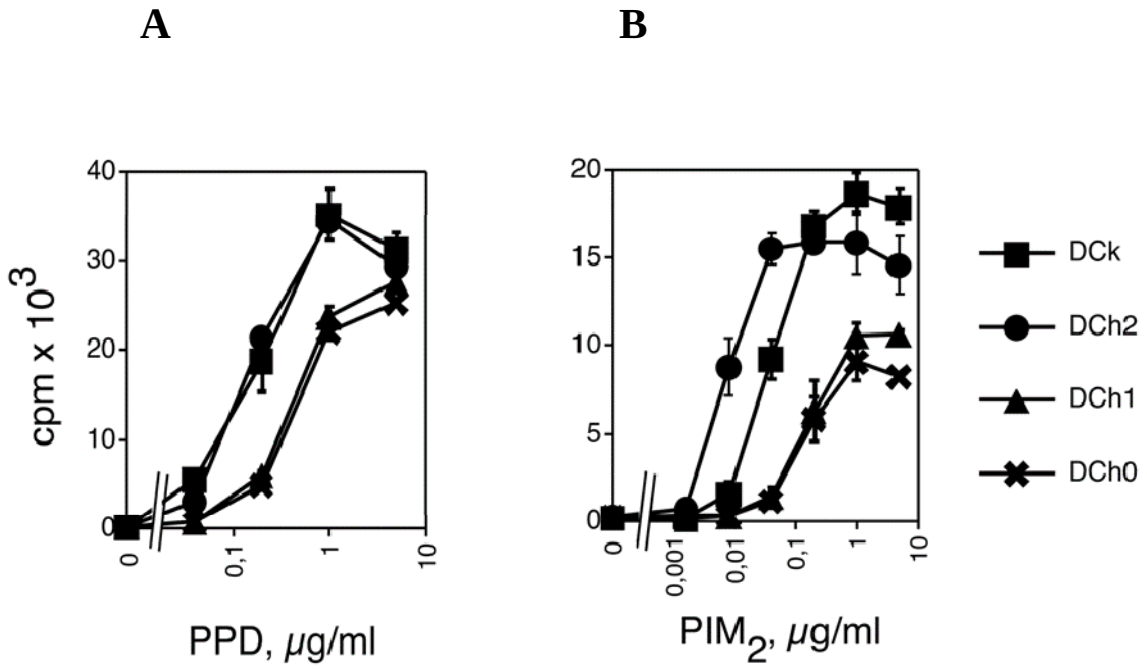


Fig 28. DC populations differentiated upon T cell activation have different capacity to stimulate memory T cells

To test the capacity of the different DC populations to stimulate memory T cells, DCk, DCh2, DCh1 or DCh0 were used to stimulate:
A) a PPD-specific MHC II-restricted or
B) a PIM_2-specific CD1b-restricted TCC.
After 48 h of co-culturing, ^{3}H-thymidine was added and cells were harvested 18 h later.
Results are expressed as mean cpm in triplicate wells $\pm$ SD.
One experiment representative of three is shown.