

Chapter V

LPS Activated Dendritic cells: “Exhausted” or alert and waiting?

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Abstract

To initiate immune responses, dendritic cells (DCs) must first become “activated” to produce both cell surface and secreted products that stimulate and educate T cells (Steinman, 1991). It has been suggested that they become “exhausted” (or tolerant) upon activation, and refractory to further stimulation (Langenkamp et al., 2000). For example, DCs stimulated, and then restimulated with the bacterial product, Lipopolysaccharide (LPS), fail to secrete such cytokines as Tumor necrosis factor- α (TNF- α) or interleukin-12p75 (IL-12p75).

Here we shown that activated DCs are far from “exhausted”. Using a model system similar to that used to establish the phenomenon of “exhaustion”; we found that so-called “exhausted” DCs were actually able to make copious amounts of IL-12p75, TNF- α , IL-10 and a wealth of other cytokines in response to signals from activated T cells, and to some combinations of cell-surface and soluble T-cell-derived signals. For example, CD40-Ligand was a necessary but insufficient stimulus that could be complemented with IL-4 and granulocyte-macrophage colony-stimulating factor to induce IL-12p75 production from DCs.

Negative signals also existed, as, for example, T cells, that produce large amounts of IL-4 and express high levels of CD40-Ligand, were unable to stimulate the production of IL-12p75, if they also made high levels of IL-10 (T_H2 cells). These data suggest that secretion of IL-12p75 by DCs is tightly controlled. The DCs are not “exhausted” but are simply waiting for appropriate signals to specifically deliver their products to the right cells, in the right place at the right time.

Material and Methods

Mice

All mice were kept in NIH animal facilities (an AAALAC accredited facility) in an SPF barrier colony. Adult 8-14 wk old male and female B10.A RAG2KO, B10.BR were obtained from the NIAID contract facility at Taconic Farms Germantown, NY. To generate 5C.C7 RAG2KO TCR transgenic mice (specific for moth cytochrome *c* (MCC) peptide The moth cytochrome *c* (MCC) 88-103 specific 5C.C7 TCR transgenic RAG2KO mouse, C57BL/6 TCR-Cyt 5C.C7-1 transgenic mice were backcrossed multiple times to B10.A mice in the NIAID contract facility at Taconic Farms. This strain is referred to as B10.A/SgSnAi TCR-Cyt 5C.C7-1 RAG2KO. The transgenic 5C.C7 CD40LKO and IL-4KO mice were also generated at the NIAID/Taconic Farms, Inc. These mice can be purchased from the NIAID/Taconic Farms, Inc. exchange.

Media, reagents and cell lines

Bacterial LPS that was used throughout obtained from *Escherichia (E.) coli* Serotype 0127:B8 (Sigma). Recombinant mouse GM-CSF, IFN- γ were from Peprotech Inc. The medium used throughout was Iscove's Modified Dulbecco Medium IMDM (GibcoBRL). Complete culture medium consisted of IMDM supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS: that was tested for endotoxin, mycoplasma, virus and bacteriophage GibcoBRL), plus L-glutamine, 55 μ M 2 β -Mercaptoethanol (GibcoBRL), penicillin, streptomycin and gentamicin (Bisource). For the BM-DC culture the complete medium was used plus GM-CSF at 30U/ml and IL-4 at 30U/ml. T_H2 clone D10.G4.1 was purchased from ATCC and cultured with irradiated splenocytes from B10.BR or B10.A plus 100 μ g/ml Conalbumin (Sigma), these cells also could be grown in the presence of 50pg/ml rIL-1 plus 20U/ml rIL-2 (Peprotech Inc.). AE7 a T_H1 clone was carried out in

culture with rIL-2.

Generation of bone marrow derived DCs (BM-DC)

Generation of bone marrow derived DCs was as described previously, with some modifications (Gallucci et al., 1999). Briefly, bone marrow cells were flushed out of the femurs and tibias of 8-12 week old mice into complete medium and pipetted vigorously to make a single cell suspension, which was then passed through a cell strainer (70- μ m Nylon mesh; BD Falcon TM). Erythrocytes were lysed using ACK lysis buffer (Biosource) and cells were washed 2x with complete medium. At this point, BM cells were either cultured immediately or cryopreserved for later use. BM cells were cultured at 1×10^6 cells/well in a 24 well plate in a final volume of 2 ml with complete medium supplemented with GM-CSF 6 ng/ml = 30U/ml and IL-4 3ng/ml = 30U/ml (Peprotech Inc.). Starting at the 3rd day of culture, half of the medium was replaced with fresh warm medium each day. On the day 7th, the plate was chilled, and clusters of loosely adherent/suspended cells were carefully removed and used as a source of BM-DCs.

Generation of naïve T cells

Multiple spleens were obtained from 5C.C7 mice and single cell suspensions of these cells were incubated with a cocktail of monoclonal antibodies to B220 (RA3-6B2), CD11b (M1/70), CD11c, DX5, GR1 and anti class-II (I-E^k) (14.4.4S) (BD Pharmingen) followed by binding and negative selection with Dynabead (DynaL Biotech).

In some experiments, T cells were further purified by FACS sorting to >98% purity based on the staining for CD45.1PE and the absence of staining for B220, pan-Class II, I-A^k, NK1.1, CD11c, CD11b, GR1 and CD21.

In vitro generation of antigen-activated/primed 5C.C7 CD4⁺ T cells

Spleens from B10.A/SgSnAi TCR-Cyt 5C.C7-1 RAG-2KO mice were dissociated into homogenous single cell suspensions and passed through a cell strainer (70- μ m Nylon mesh; BD Falcon TM). Erythrocytes were lysed by using ACK lysis buffer (Biosource). Splenocytes were washed 3x in cold complete medium and adjusted to 1×10^6 /ml, then cultured in up-right position in a flask in complete medium plus 1μ M MCC 88-103 at 37°C with 5% CO₂, fresh medium was added after 3 days of culture. After 5 days, cultured cells were harvested, washed 2x with medium and re-cultured in the absence of peptide with 10-20 U/ml of rIL-2. The medium was replenished every 5-7 days with fresh medium plus rIL-2. For T-DC coculture experiments, these CD4⁺ T cells were selected with a CD4⁺ T cell isolation kit containing anti-CD8a, CD11b, CD45, DX5, and Ter-119 mAbs for depletion (Miltenyi Biotech.) with the addition of anti-CD11c and anti-class-II to the above mAbs cocktail (Miltenyi Biotech.). We have used 3-4 LS columns in tandem instead of using LD columns to deplete the cells. These T cells, which were >90% CD4⁺, were used at various days of culture as a source of *in vitro* primed/antigen-activated T cells.

T_H2 Skewing conditions

Multiple spleens from naïve 5C.C7 TCR transgenic mice were stimulated under T_H2 conditions by incubating with the splenocytes with 500 μ g/ml anti-IL-12 (Peprotech) 100 μ g/ml anti-IFN- γ (R&D system) and 10 μ g/ml IL-4 (Peprotech) in the presence or absence of 0.4 μ M MCC peptide (88-103) for 3 days. Then, these cultures were split and fresh medium was added, latter they were harvested, washed and Ficoll, reculture with rIL-4. To determine T_H2 polarization the above cells were sorted and cultured at

5×10^4 cells/well T_H2 cells plus 5×10^5 /well irradiated CD3 ϵ KO splenocytes and $0.1 \mu\text{M}$ MCC peptide for 48-60 hrs, CSN were tested with the help of specific ELISA for IL-4 and IFN- γ . These T_H2 polarized cells were sorted and used in coculture assay with BM-DCs to measure IL-12p75 production.

Generation of Resting, “Exhausted” and “Hammered” DCs

Six days BM-DCs cultures were either left untreated in medium alone (Resting DCs), pre-activated with LPS at a final concentration of 100-200ng/ml LPS for 21-27hrs (“Exhausted” DCs) or LPS at final concentration of 100-200ng/ml plus 100ng/ml IFN- γ for 21-27hrs (“Hammered” DCs) in a 24 well plate at day 6. The next day loosely adherent cells were harvested, washed 2x with cold medium and restimulated in various conditions. These cells were the source of IL-12p75 production in all experiments, which was determined by ELISA in various conditions.

BM-DC/NIH3T3-CD40L coculture

Various BM-DCs (Resting, “exhausted” or “hammered”) were cocultured in a 48 or 24 well plate with 5×10^5 - 1×10^6 cells/well with mock transfected or CD40L-expressing NIH-3T3 cells for 48 hrs in the presence or absence of various cytokines. CSN from these coculture assays were tested with help of specific ELISA for the presence of IL-12p75.

BM-DC/T cell coculture

Various numbers of either sorted or bead depleted (CD4 $^+$ T cell isolation kit Miltenyi Biotech.) naïve or *in vitro* cultured 5C.C7 CD4 $^+$ T cells were incubated with 2×10^4 /well of BM-DCs/well from B10.A RAGKO mice $\pm 0.1 \mu\text{M}$ MCC peptide 88-103 in triplicates in a 96 well u-bottom plate in a final volume of $200 \mu\text{l}$ /well for 48 hrs. For kinetics studies 5×10^5 T cells were cocultured with 1×10^5 BM-DCs from B10.A RAGKO mice $\pm 0.1 \mu\text{M}$ MCC peptide 88-103 in a 24 well plate in a final volume of 2 ml. The

supernatants were removed and kept frozen at -30°C before measuring the cytokines by ELISA or SearchLight Multiplex cytokine array (Pierce Biotechnology Woburn, MA). The proliferation of 5C.C7 T cells from these cocultures was determined by adding $1\mu\text{Ci}$ of $[^3\text{H}]$ thymidine for the last 12-18 hrs of the 72 hr culture period. The amount of $[^3\text{H}]$ thymidine incorporation was determined by scintillation counting. The data are expressed as the mean cpm \pm SD of triplicate wells.

IL-10 depletion from T_H2 CSN

D10.G4.1 T cell clone was incubated at 8×10^5 /well in a 24 well plate coated with $10\mu\text{g/ml}$ of anti-CD3 mAb plus 1:50 dilution of anti-CD28 ascites for 24-48 hrs. CSN obtained was filtered through a $0.22\mu\text{M}$ syringe filter. The filtered CSN was incubated with ImmunoPure plus immobilized Protein G (Pierce) that was coupled with 1mg/ml of Rat anti-mouse IL-10 or IgG1 isotype control over night at 4°C . Post incubation with anti-IL-10 or isotype control coupled beads, CNS was filtered with $0.22\mu\text{M}$ syringe filter and it was used in the coculture of BM-DCs with antigen-activated 5C.C7 T cells.

Cytokine measurements

The concentrations of cytokines in the cell-free medium or in serum were determined using cytokine-specific ELISA. In most experiments, IL-12p75 was detected using the ELISA kit from R&D system (Quantikine) with a sensitivity of 7.8 pg/ml . In some experiments the CSN obtained were sent out as a service contract to be tested by SearchLight Multiplex cytokine array (Pierce Biotechnology Woburn, MA).

Results and Discussion

Dendritic cells (DCs) are considered one of the “sentinels” of the immune system. Among various subtypes, migratory DCs are instrumental in bringing antigens from the peripheral tissue to the secondary lymphoid organs, integrating the immune responses by stimulating the naïve T cells. During this process, DCs secrete various cytokines and chemokines: one of the important cytokines secreted from antigen-bearing DCs is IL-12p75, a heterodimeric protein composed of products of two independent genes namely p35 and p40 (Trinchieri, 1995). Regulation of IL-12p75 *in vivo* is poorly understood; however, *in vitro* data suggests that secretion of bioactive IL-12p75 is the result of coordinate expression of two independent genes within the same cells (Trinchieri, 1998b).

Original observation by Beeson 1947 defined the state of endotoxin-tolerance by repeated injection of the typhoid vaccine, which indeed was followed by Freudenberg and Galanos 1988, which demonstrated that protection against a lethal challenge of Lipopolysaccharide (LPS) involves the participation of macrophages.

Recently, a study using LPS-activated-CD14⁺ DCs (incubated with LPS >8hrs) has suggested that these cells become “exhausted” and lose their IL-12p75 production upon further re-stimulation with homologous or CD40L stimulation leading to a switch from a T_H1- to T_H2-inducing cytokines. The above study concluded, that LPS-activated DCs have “exhausted” their IL-12p75 production (Langenkamp et al., 2000). This conclusion has led to the proposal that there are two independent states for DCs: early DCs that synthesize cytokine as “active” and late DCs that have lost cytokine production as “exhausted” DCs (Reiner et al., 2007).

Here we show that DCs are not “exhausted” but rather primed and waiting for the right signal(s). Previously, we found that LPS alone does not induce IL-12p75 production from bone-marrow-derived DCs (DCs). It is only during the interaction between antigen-activated T cells with antigen-bearing DCs that these cells are able to secrete complete heterodimeric IL-12p75 protein. During this study, we noticed that LPS-activated DCs that had been suggested to be “exhausted,” made IL-12p75 when they were cocultured with antigen-activated T cells with a T_H0-type cytokine profile (Abdi et al., 2006).

This was an unexpected result; it did not corroborate with the phenomenon known as DC “exhaustion”. Perhaps, one reason for this discrepancy could be that LPS in our system is not inducing IL-12p75; therefore, these cells have not lost the capacity to secrete IL-12p75 when they are re-stimulated with T cells. For this reason, we stimulated DCs under conditions that are known to be optimal for IL-12p75 production. It is well established that IFN- γ is a critical cofactor for high-level IL-12p75 production from antigen presenting cells (Abdi et al., 2006; Hilkens et al., 1997; Snijders et al., 1998; Vieira et al., 2000). Therefore, we stimulated DCs with LPS alone or LPS plus IFN- γ and then re-stimulated them again after >24-h incubation with LPS alone, LPS plus IFN- γ or antigen-activated T cells. We found that the combination of LPS plus IFN- γ induced IL-12p75 only when DCs had no prior exposure to LPS (Fig. 1A & B). It seems that LPS- or LPS plus IFN- γ -activated DCs rendered refractory to further re-stimulation with LPS or LPS plus IFN- γ , since no IL-12p75 was detected after a second stimulation (Fig. 1A & B). In contrast, antigen-activated T cells induced high levels of IL-12p75 from LPS- and/or LPS plus IFN- γ -activated DCs. These cells had already made IL-12p75 in the first

stimulation (Fig. 1 B). Unlike IL-12p75, irrespective of prior LPS- or LPS plus IFN- γ -activation, in the same culture supernatant (CSN), DCs secreted high levels of the p40 subunit in response to LPS alone (data not shown). Therefore, these DCs are not “exhausted”; they are actually making copious amounts of one subunit of the IL-12p75 heterodimer but not the intact heterodimeric protein, which requires antigen-activated T cells for its induction.

Nevertheless, we consistently found that in the presence of antigen-activated but not naïve T cells these so called “exhausted” DCS are able to secrete IL-12p75 (Fig. 1 C). Certainly, LPS- or LPS plus IFN- γ -activated DCs which have been termed “exhausted” are perfectly capable of secreting IL-12p75, yet not in response to LPS or LPS plus IFN- γ re-stimulation. But rather, they are primed and wait (alert), and if necessary, they deliver the IL-12p75 to cells with appropriate signals, which in this case is an antigen-activated T cell that has the ability to induce IL-12p75 production. This points to the importance of appropriate signaling for the IL-12p75 production that is bound to be received only during the cognate interactions between an antigen-bearing DCs and antigen specific T cells.

We reasoned that perhaps the failure of the previous study to induce IL-12p75 from LPS-activated DCs is due to the use of CD40L, which is only a component of intact T cells and therefore, CD40L by itself possibly is insufficient to induce IL-12p75. Furthermore, it has been suggested that CD4⁺ T cells “license”/“help” DCs through engagement of CD40L/CD40 in priming cytotoxic T lymphocytes (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). This engagement triggers IL-12p75 production (Koch et al., 1996), which has been termed signal-3 (Curtsinger et al., 2003). Moreover, in a study that utilized human CD4⁺ T cells from patients

that were deficient in CD40L gene, it was demonstrated that secretion of IL-12p75 was absent in monocytes-derived DCs (Miro et al., 2006).

Subsequently, we decided to use an NIH-3T3-transfected cell line with mouse CD40L in the presence or absence of LPS to test its effects in our system. It has been suggested that CD40 synergizes with microbial priming for induction of high levels of IL-12p75 (Schulz et al., 2000). We cocultured resting and LPS-activated DCs with various conditions and measured IL-12p75 secretion in the coculture SN (Fig. 2 A). Although, T cells always induced copious amounts IL-12p75 production from DCs, suggesting that DCs are not “exhausted” yet, CD40L induction of IL-12p75 was inconsistent; sometimes we were able to measure IL-12p75 and other times we could not detect any (data not shown). To determine if the reasons for these inconsistencies were due to the kinetic differences of CD40L induced IL-12p75 production, we measured IL-12p75 at various time points from DCs cocultured with NIH-3T3-CD40L or antigen-activated T cells. We found that IL-12p75 is rapidly secreted as early as 6-h from the LPS-activated DCs in both CD40L and T cells cocultured SN (Fig. 2 B). Therefore, the kinetics of IL-12p75 production was not the reason for the observed inconsistencies when CD40L was employed to stimulate DCs.

We next turned to the components of the medium as a source that might be conducive for *in vitro* production of IL-12p75, because it had been previously shown that the production of IL-12p75 could be greatly enhanced by the addition of IL-4 and GM-CSF (Hochrein et al., 2000). These are the components that are commonly used to generate DCs (from bone marrow in mouse and monocytes in human). In general, bone marrow cells are cultured in a medium containing GM-CSF and IL-4 to

differentiate them into DCs; these exogenous cytokines may remain in residual amounts that vary from experiment to experiment.

To see if these components contributed to the variation in IL-12p75 production in the presence of CD40L, we added recombinant IL-4, GM-CSF and IFN- γ or a combination of these three cytokines to the coculture of resting, LPS- or LPS plus IFN- γ -activated DCs with NIH-3T3-CD40L or antigen-activated T cells and measured their IL-12p75 production. The addition of IL-4 to CD40L DCs' coculture had little effect on resting (Fig. 2 C-i), a moderate effect on LPS-activated (Fig. 2C-ii) and a dramatic effect on LPS plus IFN- γ -activated DCs' IL-12p75 production (Fig. 2 C-iii). Moreover, when a combination of GM-CSF plus IL-4 was incorporated into a CD40L-DCs coculture, it had a great effect on resting (Fig. 2c-i), a moderate effect on LPS- (Fig. 2c-ii), and a dramatic effect on LPS plus IFN- γ -activated DCs IL-12p75 production (Fig. 2c-iii). Furthermore, the combination of IFN- γ with IL-4 plus GM-CSF led to a dramatic increase in IL-12p75 secretion from all three DCs that were cocultured with CD40L, pointing to the synergistic activities of all three cytokines with CD40L for IL-12p75 production (Fig. 2C i-iii). Yet, in parallel, T cells independently, in the absence of any cytokines, were perfectly capable of inducing IL-12p75 production from DCs (Fig. 2C i-iii). These findings imply that IL-4 and CD40L play an important role in induction of IL-12p75 from DCs.

We next evaluated the contribution of CD40L from T cells and its effect on secretion of IL-12p75 from DCs. We compared T cells from wild type or CD40LKO 5C.C7 TCR-transgenic mice in the coculture assay with DCs and found that a lack of CD40L on antigen-activated T cells renders DCs incapable of secreting IL-12p75 (Fig. 2 D) but has no effects on T cells proliferation as WT and CD40LKO 5C.C7 T cells proliferated equally under

the same culture conditions (Fig. 2 E). Our data are consistent with previous observations that CD40-CD40L is a critical contributor of IL-12p75 production from DCs (Koch et al., 1996; Wong et al., 2008).

We next asked if IL-4 secreted from antigen-activated T cells played any role in priming the DCs, instructing them to secrete IL-12p75. We took advantage of T cells from IL-4KO 5C.C7 TCR-transgenic mice in our coculture system and found that antigen-activated IL-4KO T cells are consistently impaired in inducing IL-12p75 production from DCs in comparison to their wild-type counter parts (Fig. 2 F). Although, this reduction was not as dramatic as when T cells from CD40LKO antigen-activated T cells were used. There was a 3- to 4-fold reduction of IL-12p75 production when antigen-activated T cells were obtained from IL-4KO mice when compared with wild-type T cells. Furthermore, this impairment of IL-12p75 production could be reconstituted by addition of recombinant IL-4 to the coculture of IL-4KO T cell and DCs (Fig. 2 F).

From these data, we deduced that a T_H2 cells that usually express CD40L and secrete high levels of IL-4 should be able to induce IL-12p75. We tested this premise by using classic representatives D10.G4.1 a T_H2 clone and the AE7 a T_H1 clone. To our surprise, the D10.G4.1 clone did not induce any IL-12p75 from DCs; in contrast, AE7 clone was very effective in inducing IL-12p75 (Fig. 3 A). The surface phenotype confirmed that both clones AE7 and D10.G4.1 express high levels of CD40L (data not shown). We reasoned, that perhaps there is something wrong with the long-term T_H2 clone therefore; we simultaneously generated four independent T_H2 cells by skewing them *in vitro* in classical T_H2 conditions (fig. 3 B). Again, the skewed T_H2 cells behaved like the T_H2 T cell clone; they were incapable of inducing IL-12p75 when they were cocultured with DCs (Fig. 3 B). The

ELISA confirmed that these cells are truly skewed T_H2 cells and they make signature T_H2-type cytokines, including copious amount of IL-4. Moreover, these cells express high levels of CD40L and they proliferate equally in the presence of antigen in comparison with T_H0 cells (data not shown). But the surprising issue was why they cannot induce IL-12p75?

To address this question, we asked if there is something in the SN of T_H2 cells that inhibits IL-12p75 production. Thus, we titrated the D10.G4.1 SN into the coculture of antigen-activated T cells with DCs and found that there is a dose-dependent inhibition of IL-12p75 production in the presence of SN from T_H2 clone (Fig. 3 C). With the help of a Multiplex cytokine array we simultaneously measured various cytokines that are produced by D10.G4.1 T_H2 clone in an antigen-presenting-free system. The cytokine profile obtained from D10.G4.1 SN demonstrated that in the presence of plate-bound anti-CD3, these cells are making copious amounts of T_H2-type cytokines including IL-10 (Fig. 3 D). It is well established that IL-10 is the negative regulator of IL-12p75 (Trinchieri, 1998a; Trinchieri, 2007). Therefore, to see if the inhibitory activities were due to the presence of IL-10 in the supernatant, we removed IL-10 from the SN of D10.G4.1 specifically with the help of an anti-IL-10 affinity purification column. Once this was accomplished, when we re-tested IL-10 depleted SN in the coculture of antigen-activated T cells with DCs, we were able to reconstitute IL-12p75 activities. In contrast, the control SN that was depleted with an isotype control, which still contained IL-10, inhibited IL-12p75 production when added to the coculture assay (Fig. 3 E-i). Furthermore, by using a specific ELISA that measures IL-10, we confirmed that IL-10 was specifically removed from the D10.G4.1 SN

(Fig. 3 E-ii). This further supports the notion that the presence of IL-10 is directly correlates with the inhibition of IL-12p75 production (Fig. 3 E-i). Moreover, addition of ³H-thymidine into the same wells indicates that there is no defect in T cells as they proliferated normally in all conditions irrespective of the presence or absence of IL-12p75 or IL10 (Fig. 3 E-iii).

These data collectively demonstrate a complex interplay between antigen-activated T cells and antigen-bearing DCs, the signals that are exchanged between them and the cytokine milieu that these cells are interacting with each other. Our data specifically demonstrates that the LPS-activated DCs are not “exhausted” but rather are waiting for the appropriate signals exchanged between the antigen-bearing DCs and antigen-specific T cells.

It has been shown that IL-12p75 is the major cytokine to induce T_H1-type responses (Heufler et al., 1996; Hsieh et al., 1993). Thus, it is currently believed that during the primary immune response, interactions between antigen-bearing DCs with naïve T cells should create an environment conducive for IL-12p75 production, in which this cytokine will skew the immune response toward a T_H1 as opposed to T_H2 polarization. However, our data (compilation of 44 independent experiments) suggests that it is unlikely that naïve T cells are instrumental in inducing IL-12p75 from antigen-bearing DCs leading to the initial T_H1-type cytokine milieu (Fig. 1 C). In contrast, antigen-activated T cells encountering antigen-bearing DCs make both T_H1- and T_H2-type cytokines (manuscript in preparation). These cells are commonly known as T_H0 cells, and as long as they have not been skewed toward T cells that make high levels of IL-10, these antigen-specific T cells are perfectly competent in inducing IL-12p75 from appropriate antigen-bearing DCs (Fig. 1 B-C).

Our data further suggests that the exchange of information between antigen-bearing DCs, antigen-specific T cells and their cytokine milieu is a crucial determinant in the outcome of the immune response. Furthermore, once DCs encounter microbes, they shut down their response toward further stimulation by microbes or their products. They retain the information and wait; these cells are not “exhausted” but perfectly capable of making various cytokines including IL-12p75. The DCs are not “exhausted” but are simply waiting for the appropriate signals to specifically deliver their products to the right cells, in the right place at the right time. Nonetheless, this depends on appropriate external signals that are received by DCs from the tissue or during cognate interaction with various T cells in the lymph node by which they create a suitable milieu that is favorable for both T cells and DCs.

References

- Abdi, K., Singh, N., Matzinger, P., 2006. T-cell control of IL-12p75 production. *Scand J Immunol* 64, 83-92.
- Bennett, S.R., Carbone, F.R., Karamalis, F., Flavell, R.A., Miller, J.F., Heath, W.R., 1998. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* 393, 478-480.
- Curtsinger, J.M., Lins, D.C., Mescher, M.F., 2003. Signal 3 determines tolerance versus full activation of naive CD8 T cells: dissociating proliferation and development of effector function. *J Exp Med* 197, 1141-1151.
- Gallucci, S., Lolkema, M., Matzinger, P., 1999. Natural adjuvants: endogenous activators of dendritic cells. *Nat Med* 5, 1249-1255.
- Heufler, C., Koch, F., Stanzl, U., Topar, G., Wysocka, M., Trinchieri, G., Enk, A., Steinman, R.M., Romani, N., Schuler, G., 1996. Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-gamma production by T helper 1 cells. *Eur J Immunol* 26, 659-668.
- Hilkens, C.M., Kalinski, P., de Boer, M., Kapsenberg, M.L., 1997. Human dendritic cells require exogenous interleukin-12-inducing factors to direct the development of naive T-helper cells toward the Th1 phenotype. *Blood* 90, 1920-1926.
- Hochrein, H., O'Keeffe, M., Luft, T., Vandenabeele, S., Grumont, R.J., Maraskovsky, E., Shortman, K., 2000. Interleukin (IL)-4 is a major regulatory cytokine governing bioactive IL-12 production by mouse and human dendritic cells. *J Exp Med* 192, 823-833.
- Hsieh, C.S., Macatonia, S.E., Tripp, C.S., Wolf, S.F., O'Garra, A., Murphy, K.M., 1993. Development of TH1 CD4+ T cells through IL-12 produced by Listeria- induced macrophages [see comments]. *Science* 260, 547-549.
- Koch, F., Stanzl, U., Jennewein, P., Janke, K., Heufler, C., Kampgen, E., Romani, N., Schuler, G., 1996. High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10 [published erratum appears in *J Exp Med* 1996 Oct 1;184(4):following 1590]. *J Exp Med* 184, 741-746.
- Langenkamp, A., Messi, M., Lanzavecchia, A., Sallusto, F., 2000. Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. *Nat Immunol* 1, 311-316.

- Miro, F., Nobile, C., Blanchard, N., Lind, M., Filipe-Santos, O., Fieschi, C., Chapgier, A., Vogt, G., de Beaucoudrey, L., Kumararatne, D.S., Le Deist, F., Casanova, J.L., Amigorena, S., Hivroz, C., 2006. T cell-dependent activation of dendritic cells requires IL-12 and IFN-gamma signaling in T cells. *J Immunol* 177, 3625-3634.
- Reiner, S.L., Sallusto, F., Lanzavecchia, A., 2007. Division of labor with a workforce of one: challenges in specifying effector and memory T cell fate. *Science* 317, 622-625.
- Ridge, J.P., Di Rosa, F., Matzinger, P., 1998. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* 393, 474-478.
- Schoenberger, S.P., Toes, R.E., van der Voort, E.I., Offringa, R., Melief, C.J., 1998. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 393, 480-483.
- Schulz, O., Edwards, A.D., Schito, M., Aliberti, J., Manickasingham, S., Sher, A., Reis e Sousa, C., 2000. CD40 triggering of heterodimeric IL-12 p70 production by dendritic cells in vivo requires a microbial priming signal. *Immunity* 13, 453-462.
- Snijders, A., Kalinski, P., Hilkens, C.M., Kapsenberg, M.L., 1998. High-level IL-12 production by human dendritic cells requires two signals. *Int Immunol* 10, 1593-1598.
- Steinman, R.M., 1991. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* 9, 271-296.
- Trinchieri, G., 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu Rev Immunol* 13, 251-276.
- Trinchieri, G., 1998a. Immunobiology of interleukin-12. *Immunol Res* 17, 269-278.
- Trinchieri, G., 1998b. Interleukin-12: a cytokine at the interface of inflammation and immunity. *Adv Immunol* 70, 83-243.
- Trinchieri, G., 2007. Interleukin-10 production by effector T cells: Th1 cells show self control. *J Exp Med* 204, 239-243.
- Vieira, P.L., de Jong, E.C., Wierenga, E.A., Kapsenberg, M.L., Kalinski, P., 2000. Development of Th1-inducing capacity in myeloid dendritic cells requires environmental instruction. *J Immunol* 164, 4507-4512.
- Wong, K.L., Lew, F.C., MacAry, P.A., Kemeny, D.M., 2008. CD40L-expressing CD8 T cells prime CD8alpha(+) DC for IL-12p70 production. *Eur J Immunol* 38, 2251-2262.

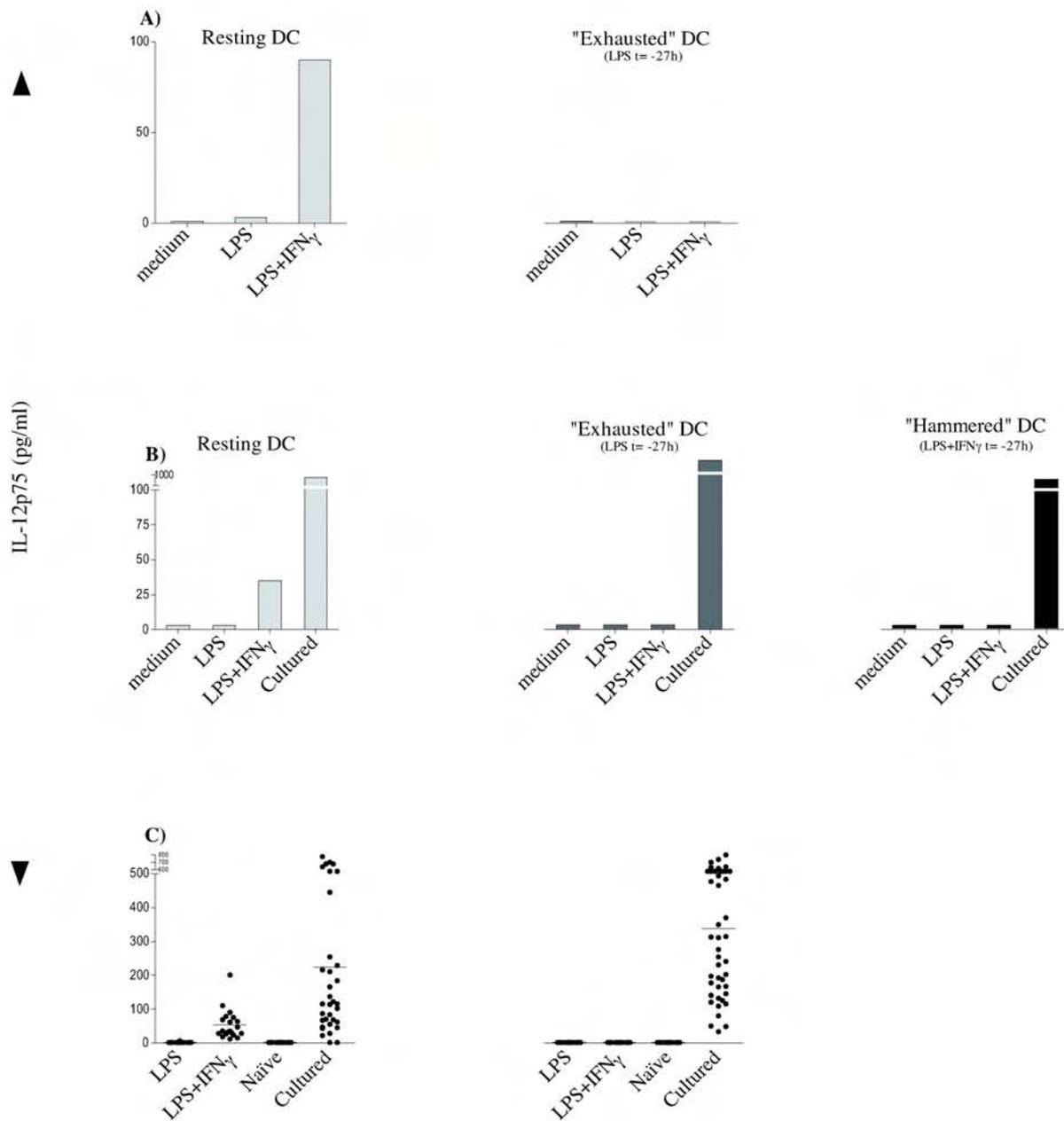


Figure 1. LPS pre-activated DCs are not “exhausted” they make IL-12p75 in the presence of antigen-activated T cells but not LPS plus rIFN- γ or naive T cells. (A) BM-DCs from B10.A-Rag2KO mice were either at resting state or pre-activated with 100ng/ml LPS for 27hr then washed and cultured at 2×10^4 cells/well in a 96 well U-bottom plate. Then these cells were restimulated with 100ng/ml LPS in the presence or absence of 100ng/ml of rIFN- γ for 48hr. CSN was tested for the presence of IL-12p75 with specific ELISA. (B) Same as (A) except one group of BM-DCs (“Hammered”) were pre-activated with 100ng/ml LPS plus 100ng/ml rIFN- γ for 27hr and then washed before being restimulated with LPS, LPS plus IFN- γ or cocultured with 1×10^5 /well antigen-activated 5C.C7 T cells plus $0.1 \mu\text{M}$ MCC peptide. (C) Same as (A) except “exhausted” BM-DCs were pre-activated with LPS for 24hr prior to coculture with naive or antigen-activated T cells in the presence of $0.1 \mu\text{M}$ MCC peptide. Each dot represents an independent experiment (compilation of 43 experiments).

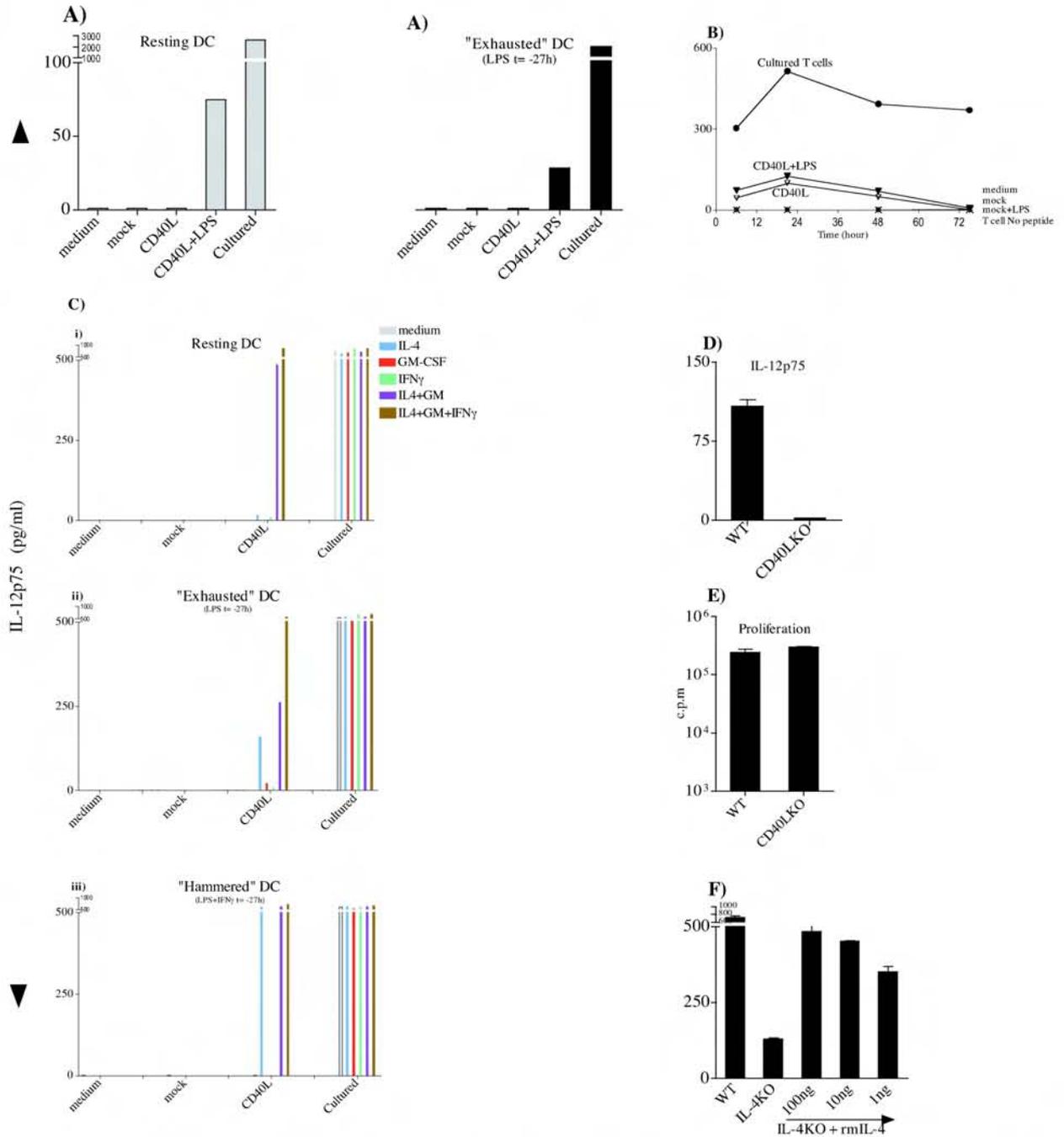


Figure 2. Combination of CD40L and IL-4 plays an important role in IL-12p75 production from BM-DCs. (A) BM-DCs from B10.A-Rag2KO mice were either at resting state or pre-activated with 100ng/ml LPS for 27hr then washed and cocultured at 1×10^5 cells/well with 5×10^5 cell /well NIH-3T3 mock, NIH-3T3 CD40L expressing cells in the presence or absence of 100ng/ml LPS or antigen-activated 5C.C7 T cells plus 0.1 μ M MCC for 48hr in a 48 well plate. CSN was tested for the presence of IL-12p75 with specific ELISA. (B) Kinetics of IL-12p75 secretion was done in the same conditions as (A) except BM-DCs were pre-activated with 100ng/ml LPS for 27hr then washed and cocultured under various conditions. At different time point CSN were collected and tested for the presence of IL-12p75 with specific ELISA. (C i-iii) Same as (A) except one group of DCs ("Hammered") were pre-activated with LPS plus rIFN- γ for 27h then washed prior to the coculture with various conditions, different cytokines were added at final concentration of 100ng/ml in a 48 well plate. CSN were tested after 48hr coculture for the presence of IL-12p75 with specific ELISA. (D) BM-DCs from B10.A-Rag2KO mice were pre-activated with 100ng/ml LPS for 27hr then washed and cocultured at 2×10^4 cells/well with 1×10^5 WT or CD40L 5C.C7 T cells plus 0.1 μ M MCC peptide in a 96 well U-bottom plate for 48 hr. CSN were tested for the presence of IL-12p75 with specific ELISA. (E) Same as (D) 3H-Thymidine incorporation was measured during the last 12 hrs of the coculture assay. (F) Same conditions as (D) except IL-4KO 5C.C7 T cells were used in the presence or absence of various concentration of rIL-4. These experiments have been repeated at least three times.

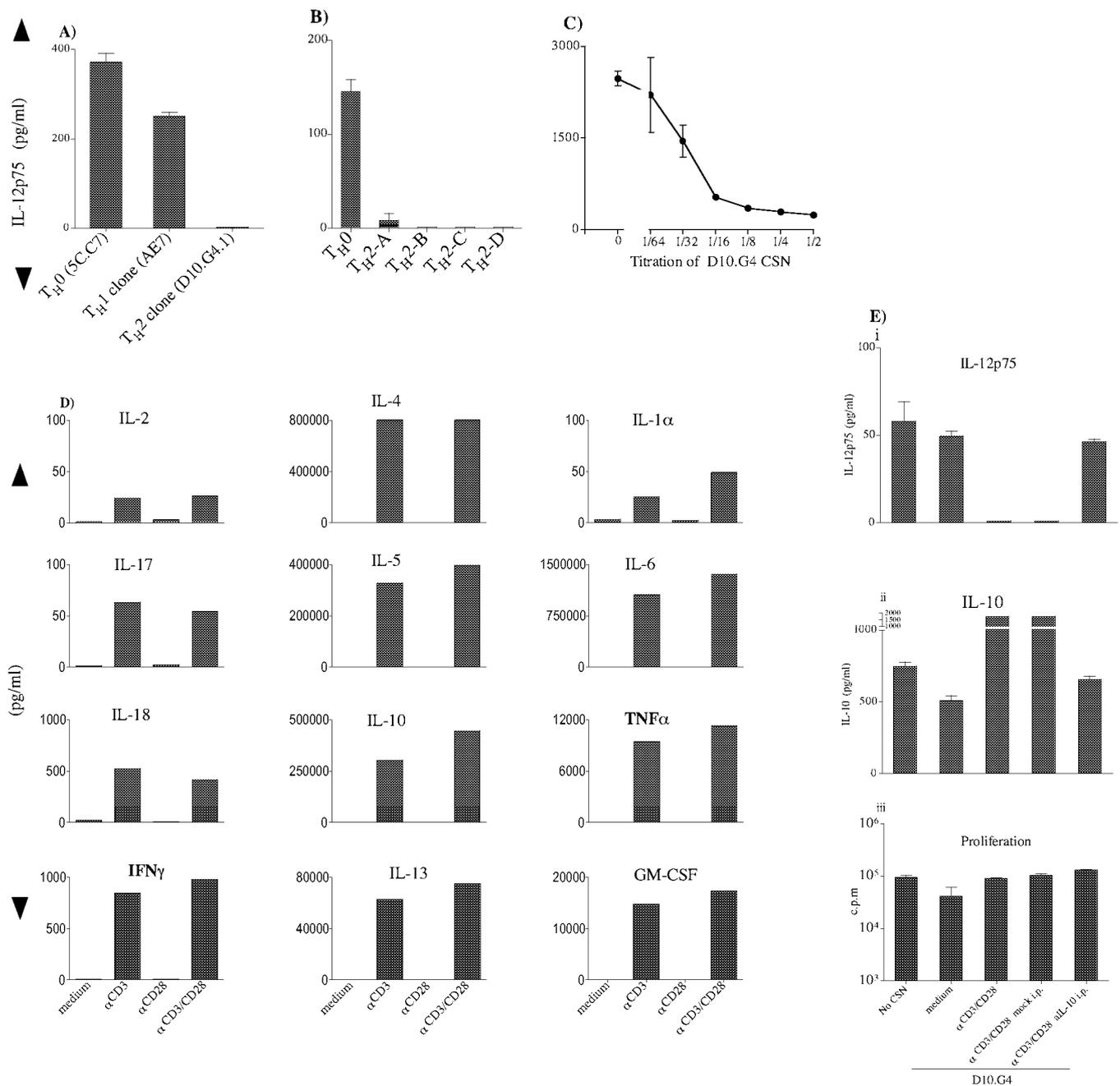


Figure 3. T_H0 and T_H1 cells induce IL-12p75 production but not T_H2 cells due to the presence of endogenous IL-10. (A) BM-DCs from B10.A-Rag2KO mice were pre-activated with 100ng/ml LPS for 24hr then washed and co-cultured at 2×10^4 cells/well with 5×10^5 cell/well 5C.C7 T cells (T_H0) AE7 (T_H1 clone, I-E^k) or D10.G4.1 (T_H2 clone, I-A^k) in the presence of $0.1 \mu\text{M}$ MCC and $1 \mu\text{M}$ conalbumin respectively for 48hr in a 96 well U-bottom plate. CSN were tested for the presence of IL-12p75 with specific ELISA. (B) Same as (A) except naïve 5C.C7 T cells were cultured under TH2 skewing conditions by adding $10 \mu\text{g/ml}$ anti-IFN γ , $10 \mu\text{g/ml}$ anti-IL-12 and 100ng/ml rIL-4 plus $0.4 \mu\text{M}$ MCC peptide for 3 days washed, Ficoll and maintained in 20U/ml rIL-2 plus 100ng/ml rIL-4, these skewed T_H2-type T cells were sorted for coculture with BM-DCs. CSN were collected and tested for the presence of IL-12p75 with specific ELISA. (C) Same as (A) antigen-activated 5C.C7 T cells were co-cultured with LPS pre-activated BM-DCs plus $0.1 \mu\text{M}$ MCC in the presence of titrated CSN obtained from APC free D10.G4.1 TH2 T cell clone that has been stimulated with plate bound anti-CD3 and soluble anti-CD28 for 48hrs in a 24 well plate. (D) Same CSN that was used in (C) was analyzed with the help of multiplex cytokine array for measuring various cytokines in CSN. (E i-iii) Same conditions as (C) except some wells received CSN from anti-CD3 stimulated D10.G4.1 or the same CSN in which endogenous IL-10 has been specifically depleted by incubating the D10.G4.1 CSN with protein-G beads coupled with Rat anti-IL-10 monoclonal antibodies or the isotype control. (i) After 48hr of co-culture assay, CSN was tested for the presence of IL-12p75 with specific ELISA. (ii) Same conditions as (i) except CSN were tested for the presence of IL-10 with specific ELISA. (iii) ³H-Thymidine incorporation was measured during the last 12 hrs of the coculture assay. These data are expressed as the mean \pm SD triplicates from a 96 well.