



A mixture of bacterial mechanical lysates is more efficient than single strain lysate and of bacterial-derived soluble products for the induction of an activating phenotype in human dendritic cells

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ABSTRACT

Dendritic cells (DCs), following an optimal maturation, are able to drive an efficient immune-response. For this, both co-stimulatory molecules (CD80 and CD86), activation molecules (CD83) and peptide presenting molecules (HLA) are over-expressed. The *in vitro* treatment of immature DC with fragments of bacterial strains, obtained by using a mechanical lysis as well as with bacterial-derived molecules (such as lipopolysaccharide and protido-glycan), induced the maturation of DCs and the secretion of a panel of cytokines and chemokines. Of note, *ex vivo* treated circulating DCs and plasmacytoid DCs were also activated by these bacterial bodies. However, while the particulate fraction of single bacterial strains or soluble bacterial-derived molecules induced a sub-optimal maturation (as evaluated by the expression of an activating phenotype on DCs and the amount of cytokine secretion), the addition of the mixture of the particulate fractions of the different bacterial strains was able to mediate an optimal maturation. These results were also confirmed by using the secretion of both cytokines and chemokines as markers of DC activation. All these findings suggest that the particulate fraction of bacterial lysate mixtures, because of their ability to interact with different surface structures, might be exploited not only as an immunogen, but also as an adjuvant treatment to boost an immune-response to poorly "antigenic" proteins, such as cancer antigens or allergens.

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1. Introduction

Dendritic cells (DCs) play a central role in the activation of the immune response. In their immature form, DC can be isolated in the periphery as well as in tissues, such as mucosae and are devoted to perform a continuous sampling of the environment. Following the engulfment of microbial antigens and in the presence pro-inflammatory cytokines, DCs are activated. While in the former state they have the capacity of acquiring exogenous materials by endocytosis, in the latter-mature- form, DCs are able not only to process but also to more efficiently present the environmental antigens to T cells. To prime naive T cells, DCs move to secondary lymphoid organs, where an efficient immune-response is driven [1]. Besides these well known properties, DCs have a central role in

the control of the immune response: indeed, they have the capability to control the expansion and activation of natural killer (NK) cells [2] which, in turn, mediate the lysis of immature DCs [3]. The biological effect of DCs in the presence of a particulate or a soluble antigen has been extensively studied: indeed, a particulate antigen obtained by apoptotic bodies of EBV infected B cells may prime T cells, while a soluble antigen obtained by the same cells was apparently less effective in order to pulse effector cells [4]. This finding was also reinforced by the observation that the regulatory circuit involving NK cells and DCs, was effectively enhanced by the presence of pathogens, such as living *Escherichia coli* or *Bacillus Calmette-Guérin* (BCG) [5]. Bacterial lysates have been introduced in the prophylaxis of infections in humans since the beginning of the past century. The original aim of treatment based on bacterial-derived antigens was the induction of an antibody response against pathogens of different organs, in particular upper and lower respiratory tract infections and urinary tract infections. Despite the extended empirical use of such an approach, little is presently known about the fine mechanisms of action of this treatment. On the basis of what is presently known about the physiology of den-

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dritic cells, it may be expected that these bacterial antigen-based drugs should be able to mediate the maturation of dendritic cells because of the presence, in the bacterial lysate, of a suitable amount of structures acting as ligands of the Pattern Recognition Receptor system [6], expressed on circulating monocyte and immature DCs. In this context, it has been shown that an oral immunostimulant constituted by chemically lysed bacteria of the respiratory tract is also able to mediate the maturation of DCs [7]. Working on the capacity of respiratory infections to induce an IgA-mediated immune-response specific for surface structure of bacteria, we have previously demonstrated that the weak naturally induced IgA secretion at salivary level could be significantly increased by the stimulation of mouth mucosa immunity with a Polyvalent Mechanical Bacterial Lysate (PMBL) [8]. This is a commercial drug containing 7 mg/capsule of a mixture of microbial bodies obtained from mechanically killed bacteria, which included *Staphylococcus aureus* (Sa), *Streptococcus pneumoniae* (Spn), *Streptococcus pyogenes* (Spy), *Hemophilus influenzae* (Hi), *Moraxella catarrhalis* (Mc) and *Klebsiella pneumoniae* (Kp). Despite the clear evidence of a de novo secretion of anti-bacterial body IgA, at present the mechanism of action of such an approach is only partially clarified. Specifically, little is known on the role of the particulate fraction, constituted by bacterial bodies, in activating and directing the immune response towards the production of a powerful mechanism of defense. This is represented by opsonizing IgA antibodies, specific for each bacterial surface structures (manuscript submitted). In addition, little is known on the contribution of a single bacterial strain in terms of effects on innate immunity, such as triggering of DC activation. In this report, we will analyze the capacity of the single bacterial strains versus the bacterial strain mixture to induce the maturation. In particular, we will describe the expression of an activating phenotype and secretion of relevant cytokines and chemokines, on different DC subsets, which include monocyte-derived DC (mo-DC), circulating myeloid DCs (cMDCs) and plasmacytoid DCs (PDCs).

2. Material and methods

2.1. Generation of mo-DCs

Whole blood from volunteers was collected by venipuncture and stored in an heparinized tube. Following a centrifugation on a discontinuous gradient of Ficoll-Paque (Amersham Pharmacia Biotech), Peripheral Blood Mononuclear Cells (PBMC) were obtained. The isolation of CD14⁺ cells (virtually only monocytes) was performed using α CD14-MicroBeads, MS+/RS+ columns, and a MiniMACS separator (Miltenyi Biotec). mo-DCs were obtained as described in [2]. Briefly, 500,000 CD14⁺ cells/ml were plated in 6-well plates with RPMI-1640, plus 1% single donor plasma, glutamine, and gentamicin. rhIL-4 (Euroclone) and rhGM-CSF (Euroclone) were added to a final concentration of 500 and 1000 U/ml, respectively, at day 0, 2, and 4. On day 5, the immature mo-DCs obtained by this first culture, were transferred to new plates at 300,000 cells/ml and cultured for two other days in the presence of either LPS (0.5 μ g/ml, Sigma), protido-glycan (5 μ g/ml, Sigma), BCG (10 μ g/ml, Organon) or the bacterial combinations. Of note, these were the optimal concentrations of soluble stimuli obtained in a preliminary set of experiments for the induction of a complete phenotypic activation of cultured immature DCs.

2.2. Bacterial lysates for DC maturation

Six different bacterial strains, representing the prototype of pathogens related to respiratory infections (Sa, Spn, Spy, Hi, Mc and Kp, kindly provided by Lallemand, Toulouse, France) were tested at different concentrations (from 0.1 to 20 μ g/ml) to induce the

maturation of mo-DCs. In addition, the single lysates (at 10 μ g/ml) were also tested. Finally, the mechanical bacterial lysate supernatant from the combination of the six strains, was obtained after an overnight incubation of 0.5 g in 5 ml of water and used as control.

2.3. Flow cytometric analysis

To analyze the capacity of an exogenous stimulus (such as a bacterial lysate or a soluble bacterial-derived protein) to induce the maturation of DCs, five different surface molecules (CD1a, CD80, CD83, CD86 and HLA class II) directly involved in the capacity of DCs to prime naive T cells, were used. HLA class II is well known to be the surface structure involved in the presentation of exogenous peptides, derived from proteins engulfed by immature DC and then processed at intracellular level. CD80 and CD86 are the co-stimulatory molecules that stabilize and potentiate the antigen-related signal to lymphoid cells. In addition, the de novo expression of CD83 (an activation marker of DC) was also employed. The analysis of DC surface markers on live cells was performed by using the following monoclonal antibodies (mAbs) in immunofluorescence assays: FITC-conjugated CD1a, PE-conjugated anti-CD86, FITC-conjugated anti-CD80, FITC-conjugated anti-CD83, PE-conjugated anti-CD83 (from Beckman Coulter, Milano, Italy). Direct immunofluorescence procedure was performed by diluting fluorochrome-labeled mAb with 1 mg/ml human γ -globulin (human therapy grade from a commercial source), in order to block nonspecific Fc-receptor binding. Cells were then washed and the flow cytometric analysis was performed. Negative controls included directly labeled or unlabeled isotype-matched irrelevant mAbs. Cells were then washed and analyzed by flow cytometry. Flow cytometric analysis was performed on a FAC-SCanto flow cytometer (Becton Dickinson, Milano, Italy). Results were then expressed as Mean Fluorescence Intensity (MFI).

2.4. Circulating dendritic cells

In an attempt of defining whether other DCs can be induced to an activating phenotype by the addition of bacterial lysates, cMDCs were identified as mononuclear cells (CD3,19,20, 56, 14 negative, DR⁺ and CD11c⁺ cells), while pDCs were also identified as CD3,19,20, 56, 14 negative, DR⁺ and CD11c⁺ negative cells. The capacity of different bacterial lysate to mediate the activation of cMDCs was analyzed by adding 10 μ g/ml of the different bacterial lysates to 0.05 ml of whole peripheral blood. CD80, CD83 and CD86 expression were measured on these cells in order to detect the activation of circulating cMDCs. For pDCs, the absence of surface structures like TLR2 and TLR4, virtually ruled out the possibility that a direct activation of these cells may occur. Nevertheless, the secretion of cytokines by cMDCs could indirectly induce pDCs activation. For this reason, also pDCs were evaluated using the above-mentioned antibodies and flow cytometry.

2.5. Analysis of cytokine and chemokine secretion

Cytokine and chemokine concentrations in the supernatants of immature mo-DC and of mo-DC matured under the abovementioned stimuli, were determined with the Cytokine and Chemokine Bead Array (CBA, BD Pharmingen, San Diego, CA) reagents using flow cytometry according to the manufacturer's instructions. Briefly, 0.05 ml of mixed capture beads were incubated with 0.05 ml samples for 1 h at room temperature and then 0.05 ml of mixed phycoerythrin detection reagent was added. After 1 h incubation, the beads were washed extensively and then analyzed using a FACSAria flow cytometer (BD Biosciences, Mountain View, CA). Reference curves for the different molecules were obtained by plotting the concentrations vs fluorescence results. The experimental results

were calculated on the regression curve. The tests were performed in triplicate. In the different experimental conditions, the secretion of the following cytokines and chemokines was evaluated. IL12p70 is a cytokine secreted by mature DCs involved in a powerful T cell priming. TNF- α is a prototype of the pro-inflammatory cytokines secreted by mature DCs, while IL10 is the prototype of regulatory cytokines. IFN- γ is secreted by mature DCs, as well as IL1b, IL-2 and IL-6. All these secreted molecules could act reinforcing the maturation of DCs and the acquisition of an activated phenotype. To these molecules, it should be added CXCL9, CXCL10, CCL2, CCL5 (RANTES) and IL8, a group of chemokines that can significantly modify the capacity of DCs to migrate to the secondary lymphoid organs and recruit other effector cells such as granulocytes. [9,10].

2.6. Analysis of the concentration of endotoxins using the *Limulus Amebocyte Lysate* (LAL) test

Finally, in an attempt define whether the capacity of inducing the maturation of DCs was related to the mere presence of LPS in bacterial lysates, the endotoxin content of the different lysates as well as of the mixture was analyzed using the LAL test. Each lyophilized mechanically-lysed microorganism and the mixture of the six main components was resuspended and diluted 1:1000 in sterile saline at the final concentration of 1 μ g/ml (weight/volume). All LAL reagents, endotoxin-free water, endotoxin-free pipette tips, endotoxin-free test tube, and endotoxin-free microplates were purchased from PBI International (Milan, Italy). Total endotoxin was measured using a kinetic chromogenic LAL test (Associates of Cape Cod Inc.) according to the manufacturer's instructions [11]. Briefly, a standard curve was plotted by using different concentrations (0.005, 0.05, 0.5, 5 and 50 EU/ml) of control standard endotoxin (CSE, *E. coli* 0113:H10). 0.05 ml of undiluted samples were added in duplicate in the 96-well microplate with the respective positive controls, mixed with 0.05 ml of LAL reagent and incubated at 37 °C. The endotoxin content was measured at 405 nm, as the chromogenic response to a chromophore, using the Gen5™ microplate software (BioTek Instruments Inc., Winooski, VT, USA), which calculated endotoxin dose (in EU/ml) starting from colour-development rate. To further validate the assay, the endotoxin recovery was also determined according to Park et al. [12].

3. Results

Different bacterial lysates were used to induce the maturation of mo-DC in an in vitro model. Table 1 shows the result of the dose-effect titration of this capacity. A clear dose-response is evident although at a PMBL concentration higher than 10 μ g/ml, no further increase of surface molecules was observed (not shown). While CD1a remained unmodified, both CD80 and CD86 were up-regulated following maturation in the presence of the six different components of the bacterial lysate. Of note, as shown in Table 2, Spn and Spy, at the dose of 10 μ g/ml, were the weaker inducers of DC activation. Sa, Kp and Mc had a four to five fold greater capacity of activating DC, while Hi was the stronger inducer of a DC activating phenotype. According to these results, the capacity of single microbial strains to activate DCs seemed to be extremely heterogeneous, even if the concentration of bacterial lysate (10 μ g/ml) was always the same. Three different experiments, in which the mixture containing all the six strains was used, are also shown in Table 2. The capacity of the mixture to induce an activating phenotype characterized by a stronger expression of surface molecules is evident. To evaluate the role of the particulate antigens in the activation of DCs, the bacterial mixture supernatant, obtained by eluting the soluble fraction of the whole bacterial lysate, was assayed. As shown in Table 3, the supernatant was much less effective than the par-

ticulate fraction in the induction of DC maturation. Other soluble molecules, used as controls, were also tested. Both LPS and PG, used at the optimal concentrations, were from 3 to 6 fold more active than the mixture supernatant. Finally, BCG, already shown to be a powerful maturation stimulus for DCs, was as efficient as both PG and LPS, but less efficient than the particulate mixture of bacteria used in these experiments. In an attempt to better define the maturation induced by single mechanical bacterial lysates, by the mixture of lysates and by the soluble counterparts, relevant cytokine and chemokine secretion was evaluated. Table 4 shows the results of a representative experiment. Of note, virtually all analyzed cytokines and chemokines studied resulted secreted in large amounts, with the exception of IL-10 and IFN- γ . The large amounts of IL12p70 and IL-2 (most likely due to mature DC-mediated T cell activation) was suggestive of a maturation of DCs that may support an efficient T cell priming. In addition, although not tested, the presence of these cytokines in the supernatant of mo-DCs suggests also that a similar behavior may be observed in blood DC. Remarkably, following treatment of peripheral blood mononuclear cells with the bacterial lysate, the number of activated, CD83+ cMDCs was significantly increased (Table 5). Of note, in the same set of experiments, also activated pDCs increased, although their number was lower than activated cMDCs. Finally, as expected, lysates of gram-negative bacteria had measurable endotoxin concentrations (Hi, Kp and Mc resulted 1.56, 1.29 and 6.10 EU/ml, respectively). On the contrary, only Spy lysates showed an extremely low concentration of endotoxins (0.058 EU/ml), while Sa and Spn lysates resulted completely negative. In addition, the mixture of the six different strains resulted 3.12 EU/ml. These results indicated that the acquisition of an activated phenotype in both mo-DCs, but also pDC and cMDCs is not only related to the presence of LPS (as a prototype of endotoxins) in the bacterial lysates and in the soluble stimuli used in these experiments.

4. Discussion

Since the discovery of the expression of a panel of Pattern Recognition Receptors, including the Toll-like Receptor system, on innate immunity cells, it was well known that components of bacterial origin may induce DC maturation. Thus, a mechanical bacterial lysate should be expected to act as DC activating stimulus. In our experiments, it has been observed that specific differences might exist by using distinct bacterial components. For instance, Spy was less effective than Mc and Hi in the capacity of inducing the expression of CD80, CD83, CD86 and HLA class II. The evidence that different microbes may express different capacities in modulating DC functions has already been observed. Indeed, different *Lactobacillus* strains were characterized by a different capacity of stimulating DCs [13,14]. Interestingly, while some microbes (such as Mc and Hi) were particularly active in their capacity of inducing the expression of an activated phenotype on DCs, the mixture of all six different microbes resulted in an even more powerful induction. A mechanistic explanation of this finding, related to simple dose-response effect, can be ruled out because the amounts of bacterial bodies were always highly comparable (a total of 10 μ g/ml). On the contrary, the synergistic effect caused by the heterogeneity of interactions related to the presence of different microbial strains, could be responsible of the increased induction of activation pathways, resulting in a greater expression of co-stimulatory molecules. Then, soluble stimuli (represented by PG, LPS and BCG) used at doses able to induce the highest expression of co-stimulatory molecules, were less effective than the mixture of bacterial lysates. Even in this case, a mechanistic explanation can be provided using the above-mentioned reasons. Nevertheless, it might rather be that bacterial fragments, obtained through mechanical lysis and

Table 1

Expression of CD80 and CD86 in mo-DCs treated with different doses of bacterial lysates. Numbers express Mean Fluorescence Intensity (MFI).

	Immature	0.1 µg/ml	0.3 µg/ml	1.0 µg/ml	3.0 µg/ml	10 µg/ml
Median CD80	2.3	2.9	5.8	6.1	7.2	7.8
Range	0.6–3.5	0.9–5.2	2.9–7.2	4.6–8.7	5.2–10.4	5.8–11.6
Median CD86	3.0	5.1	12.0	36.1	75.3	105.4
Range	1.2–4.8	0.6–14.2	0.5–26.8	24.1–43.3	35.8–93.2	48.6–136.1

Table 2

Expression of surface molecules on mo-DCs treated with different stimuli. Numbers express Mean Fluorescence Intensity (MFI).

	Immature DC				Mature DC				Ratio				Sum
	CD80	CD83	CD86	DR	CD80	CD83	CD86	DR	CD80	CD83	CD86	DR	
Spn	3.22	0.59	2.78	40.40	3.37	0.59	4.17	42.40	1.05	1.00	1.50	1.05	4.60
Range	1.7–3.5	0.4–0.8	2.1–3.1	35.0–42.1	3.1–4.05	0.4–0.65	3.7–4.8	31.8–48.2					
Spy	3.40	0.70	2.70	36.20	3.37	0.51	6.77	55.00	0.99	0.73	2.51	1.52	5.74
Range	1.2–3.4	0.3–1.8	2.0–3.8	28.0–44.1	3.2–4.45	0.3–0.75	3.3–9.2	28.8–59.1					
Sa	3.60	0.45	2.41	41.30	4.38	0.96	25.80	91.00	1.22	2.13	10.71	2.20	16.26
Range	1.6–4.4	0.4–0.9	1.0–3.6	36.1–48.2	2.9–4.25	0.3–1.2	20.1–28.3	83.4–108.7					
Kp	2.80	0.60	2.94	31.80	5.65	1.60	44.30	122.00	2.02	2.67	15.07	3.84	23.59
Range	1.5–3.6	0.3–0.7	2.2–3.3	33.4–38.1	4.1–7.35	0.8–2.3	41.0–48.2	110–131					
Mc	3.22	0.65	3.01	44.10	6.06	2.26	55.20	171.20	1.88	3.48	18.34	3.88	27.58
Range	1.9–4.1	0.3–1.1	2.0–3.1	33.4–46.1	5.1–6.5	0.8–2.7	48.2–62.8	154–184					
Hi	2.28	0.44	2.45	40.30	7.30	3.22	72.40	55.20	3.20	7.32	29.55	1.37	41.44
Range	1.4–3.6	0.45–0.9	1.8–3.9	31.2–43.2	6.3–9.05	1.8–3.65	38.8–91.2	31.8–48.2					
PMBL	1.87	0.70	2.55	31.00	7.90	3.78	96.40	384.00	4.22	5.40	37.80	12.39	59.82
PMBL	2.11	0.55	3.59	37.40	8.55	4.31	105.00	401.00	4.05	7.84	29.25	10.72	51.86
PMBL	2.97	0.85	2.89	42.00	9.22	6.01	95.50	434.50	3.10	7.07	33.04	10.35	53.57

Spn, *Streptococcus pneumoniae*; Spy, *Streptococcus pyogenes*; Sa, *Staphylococcus aureus*; Kp, *Klebsiella pneumoniae*; Mc, *Moraxella catarrhalis*; Hi, *Hemophilus influenzae*. All bacterial strains were administered at 10 µg/ml. PMBL, a combination of Spn, Spy, Sa, Kp, Mc, Hi in identical parts for a total of 10 µg/ml. All text were performed in triplicate. The raw data of the three representative experiments for PMBL are shown. Ratio, the fold increase of each surface structure after maturation of DC; Sum, the sum of Ratios for each experimental condition.

Table 3

Expression of surface molecules on mo-DCs treated with different stimuli Numbers express Arbitrary Units of Fluorescence.

	Immature DC				Mature DC				Ratio				Sum
	CD80	CD83	CD86	DR	CD80	CD83	CD86	DR	CD80	CD83	CD86	DR	
SN	3.10	0.46	2.95	40.10	4.45	0.91	3.22	49.20	1.44	1.98	1.09	1.23	5.73
Range	2.44–3.87	0.40–0.50	2.15–3.66	38.1–48.9	3.87–5.79	0.58–1.12	2.99–4.06	34.9–61.5					
LPS	2.95	0.33	3.21	36.80	9.20	2.21	12.10	128.90	3.12	6.70	3.77	3.50	17.09
Range	2.03–3.56	0.29–0.41	3.05–3.53	29.8–46.37	8.0–12.1	1.75–2.52	9.08–14.0	96.6–144					
PG	3.24	0.56	3.58	33.80	9.36	4.48	64.20	155.80	2.89	8.00	17.93	4.61	33.43
Range	2.10–3.49	0.39–0.68	2.86–4.48	20.6–42.2	8.33–10.39	2.91–5.02	47.5–71.9	102.8–172					
BCG	2.90	0.74	3.25	44.10	12.40	3.59	58.30	161.80	4.28	4.85	17.94	3.67	30.73
Range	2.43–3.19	0.47–0.84	2.67–3.97	41.9–56.0	11.1–16.2	2.41–4.45	42.5–62.9	129–190					
PMBL	2.32	0.70	3.01	36.80	8.56	4.70	98.97	406.50	3.79	6.77	33.37	11.15	55.08
Range	1.80–2.82	0.66–0.90	2.32–3.31	34.2–44.9	5.82–9.93	3.85–5.92	79.1–116	252–451					

SN, Supernatant of an overnight incubation of PMBL in saline solution. LPS, Lipopolysaccharide from *E. coli*. PG, Protidoglycan; BCG, Bacillus Calmette-Guerin. PMBL, a combination of DP, SP, SA, KP, MC, HI in identical parts for a total of 10 µg/ml. All text were performed in triplicate. Ratio, the fold increase of each surface structure after maturation of DC; Sum, the sum of Ratios for each experimental condition.

Table 4

Secretion of cytokines and chemokines from immature mo-DCs and from mo-DCs activated using different stimuli.

	IL12p70	TNF-α	IL10	CXCL10	IFN-γ	IL1β	IL-8	IL6	CCL5	CXCL9	CCL2	IL-2
Immature DC	1	1	43	23	20	8	0	2	0	0	0	22
DP	14	65	80	81	40	19	9	55	6	16	4	66
SP	26	50	63	76	80	13	16	6	3	44	6	48
SA	100	75	79	85	60	6	53	1	19	24	8	78
KP	70	77	60	100	100	56	40	60	36	22	32	100
MC	96	71	77	100	60	36	88	75	33	57	68	59
HI	93	77	66	89	90	53	52	95	48	25	100	29
PMBL	100	100	100	100	100	100	100	100	98	100	77	98
LPS	28	56	53	38	100	27	29	78	100	80	55	48
PG	18	1	40	27	100	39	17	57	31	3	28	34
BCG	42	5	57	40	40	47	49	78	34	13	79	42
SN	6	1	44	12	40	35	3	5	8	9	16	31
Min. value	1	22	21	635	1	27	20	23	42	0	0	104
Max value	198	2474	53	5516	5	450	5264	3141	15375	744	265	464

Cytokine (IL12p70, TNF-α, IL10, IFN-γ, IL1-β, IL-8, IL6 and IL-2) and chemokine (CXCL10, CCL5, CXCL9 and CCL2) secretion in immature mo-DC and in mo-DC after maturation in the presence of different stimuli. For each cytokine or chemokine, numbers represent the highest result (100) observed and the percentage of this result in the other different experimental conditions. Of note, IFN-γ (ranging from 1 to 5 ng/ml) and IL-10 (ranging from 21 to 53 ng/ml) resulted in a very poor secretion, while the other showed concentrations in the expected ranges. The capacity of the complete mixture of bacterial lysate to induce the secretion of both chemokines and cytokines was optimal, while different stimuli resulted in a more heterogeneous capabilities.

Table 5

Expression of an activating phenotype in cMDCs and in pDC at rest and after treatment with single bacterial lysates and the mixture of the six different microbes.

	WBC	Circulating DCs	% of circulating DC	pDCs	% pDCs	cMDCs	% cMDCs
Untreated	22,100	5	0.02	1	28.6	4	71.4
AU	54,259	245	0.45	16	6.5	229	93.5
HI	51,172	841	1.64	61	7.3	780	92.7
KP	77,285	1101	1.42	164	14.9	937	85.1
MC	47,557	353	0.74	118	33.3	235	66.7
PN	65,580	227	0.35	32	14.3	195	85.7
PY	55,107	790	1.43	119	15.0	672	85.0
Mix	45,994	607	1.32	67	11.1	540	88.9

WBC, white blood cell counts used to calculate the percentages of pDCs and cMDCs. Circulating DCs, absolute number of cells characterized by the following phenotype: CD3–19–56–,CD80+83+86+DR+. pDC: cells expressing CD3–19–56–,CD80+83+86+DR+ and CD11c–; cMDCs: cells expressing CD3–19–56–,CD80+83+86+DR+ and CD11c+.

expressing highly repeated bacterial-wall related structures, acted as multiple ligands of the Pattern Recognition Receptors. These multiple interactions may be more efficient than single soluble proteins or single strain (BCG) in the induction of the expression of co-stimulatory molecules. Along this line, as stated before, a chemical bacterial lysate, containing only soluble proteins, was evaluated in the past for its capacity of inducing an activated phenotype in mature DC [7]. In that work, an upregulation of a panel of surface markers (including CD80, CD86, CD83 and HLA class II) was obtained using 10 and 100 µg/ml of stimulus. Of note, 100 µg/ml of chemical lysate were needed to achieve an upregulation of DC surface markers that was only partially comparable to that obtained using 10 µg/ml of the particulate fraction described in our work. This is an important point: indeed, from a therapeutic point of view, it should be noted that 100 µg/ml is a drug concentration that cannot be easily reached in humans administering 7 mg/day of the bacterial lysate. On the contrary, as already observed [8], the particulate bacterial fraction, derived from a mechanical lysate, was clearly able to induce a specific immune-response to bacterial antigens, thus indicating that the complete activation of the immune response had been obtained using this stimulus. Cytokine and chemokine secretion was also evaluated in the different experimental conditions: of note, the mixture of all bacterial lysates, consistently induced a more important secretion of cytokines than the single lysates and soluble counterparts. This is an important observation: indeed, while IL10, used as a prototype of regulatory cytokines, is virtually absent in the above described experimental conditions, other cytokines, directly involved in a potent T cell priming (such as IL12p70) were abundantly secreted. Furthermore, the array of chemokines secreted by monocyte-derived DCs support the hypothesis that these antigen presenting cells, following interaction with bacterial lysates, acquire the capacity of moving to secondary lymphoid organs. Finally, the secretion of IL-8, mediated by the particulate bacterial fractions, may result in a recruitment of granulocytes, an effector cell belonging to the innate immunity, with a central role in the control of bacterial infections. All these results must be carefully analyzed in the light of the experiments performed on both cMDCs and pDCs. cMDCs are extremely rare in circulating blood, but can be identified *in vivo*: of note, following stimulation with the particulate bacterial lysates, the number of activated cMDC was significantly increased. Translating the results of cytokine secretion to circulating pDC, the smaller, but evident, increase of activated pDCs in our experiments seemed to be related to the large amount of cytokines that mo-DCs (and, most likely, cMDCs) had released. Indeed, pDC are known to express intracytoplasmic Toll Like Receptors (such as TLR7 and TLR 9) and are virtually unable to engulf exogenous bacterial particles (Ferlazzo, unpublished observation). Thus, the increase of activated pDCs described in our current experiments cannot be directly related to the presence of the bacterial lysate but, rather, to the large amount of cytokines secreted by cMDC, a subset of DCs expressing surface TLRs “specific” for bacterial derived structures. In addition,

although pDC do not display surface structures for exogenous bacteria, they do have a functional Fcγ receptor: thus, *in vivo*, opsonized bacterial bodies can be also captured by pDCs that have been activated by a cMDC-mediated by-stander effect. In conclusion, it is evident that DCs, through the contact with fragments of bacteria obtained by mechanical lysis, are able to express a higher number of co-stimulatory molecules and secrete higher levels of relevant cytokines and chemokines than following incubation with a soluble counterpart. It is well known that DCs with a sub-optimal activation could be either less efficient in the activation of an immune-response, could be tolerogenic [15] or could be recognized and killed by NK cells, which are known to act as an efficient mechanism of quality control of DC-mediated immune-response [16]. The evidence that cMDCs can be rapidly induced into maturation and to present antigens, as well as that pDCs, via a by-stander effect may also be activated in an *ex-vivo* experiments, strongly supports the biological activity of particulate bacterial lysates. These particulate lysates, more than their soluble counterparts, are already recognized for their efficacy in the prevention of Respiratory Tract Infections [17] and could now be considered a good candidate to act as adjuvants in a large number of different clinical situations. These include immunotherapy of cancer employing autologous cancer lysates and, in selected cases, immunotherapy of allergy: indeed, in both cases, antigens employed as therapies may lack structures suitable to induce the maturation of DCs and, for this reason, they might be unable to drive an efficient specific immune-response.

References

- [1] Steinman RM, Idozaga J. Features of the dendritic cell lineage. *Immunol Rev* 2010;234:5–17.
- [2] Ferlazzo G, Tsang ML, Moretta L, Melioli G, Steinman RM, Münz C. Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. *J Exp Med* 2002;195:343–51.
- [3] Ferlazzo G, Semino C, Melioli G. HLA class I molecule expression is up-regulated during maturation of dendritic cells, protecting them from natural killer cell-mediated lysis. *Immunol Lett* 2001;76:37–41.
- [4] Ferlazzo G, Semino C, Spaggiari GM, Meta M, Mingari MC, Melioli G. Dendritic cells efficiently cross-prime HLA class I-restricted cytolytic T lymphocytes when pulsed with both apoptotic and necrotic cells but not with soluble cell-derived lysates. *Int Immunol* 2000;12:1741–7.
- [5] Ferlazzo G, Morandi B, D'Agostino A, Meazza R, Melioli G, Moretta A, et al. The interaction between NK cells and dendritic cells in bacterial infections results in rapid induction of NK cell activation and in the lysis of uninfected dendritic cells. *Eur J Immunol* 2003;33:306–13.
- [6] Areschoug T, Gordon S. Pattern recognition receptors and their role in innate immunity: focus on microbial protein ligands. *Contrib Microbiol* 2008;15:45–60.
- [7] Zelle-Rieser C, Ramoner R, Bartsch G, Thurnher M. A clinically approved oral vaccine against pneumotropic bacteria induces the terminal maturation of CD83+ immunostimulatory dendritic cells. *Immunol Lett* 2001;76:63–7.
- [8] Rossi GA, Peri C, Raynal ME, Defilippi AC, Risso FM, Schenone G, et al. Naturally occurring immune response against bacteria commonly involved in upper respiratory tract infections: analysis of the antigen-specific salivary IgA levels. *Immunol Lett* 2003;86:85–91.
- [9] Nagorsen D, Marincola FM, Panelli MC. Cytokine and chemokine expression profiles of maturing dendritic cells using multiprotein platform arrays. *Cytokine* 2004;25:31–5.

- [10] Remondo C, Cereda V, Mostböck S, Sabzevari H, Franzusoff A, Schlom J, et al. Human dendritic cell maturation and activation by a heat-killed recombinant yeast (*Saccharomyces cerevisiae*) vector encoding carcinoembryonic antigen. *Vaccine* 2009;27:987–94.
- [11] Martino FC, Chiesa WM, Zaia AA, Ferraz CC, Almeida JF, Souza-Filho FJ, et al. Comparison of endotoxin levels in previous studies on primary endodontic infections. *J Endod* 2011;37:163–7.
- [12] Park CY, Jung SH, Bak JP, Lee SS, Rhee DK. Comparison of the rabbit pyrogen test and Limulus amoebocyte lysate (LAL) assay for endotoxin in hepatitis B vaccines and the effect of aluminum hydroxide. *Biologicals* 2005;33:145–51.
- [13] Fink LN, Zeuthen LH, Christensen HR, Morandi B, Frøkiaer H, Ferlazzo G. Distinct gut-derived lactic acid bacteria elicit divergent dendritic cell-mediated NK cell responses. *Int Immunol* 2007;19:1319–27.
- [14] Fink LN, Zeuthen LH, Ferlazzo G, Frøkiaer H. Human antigen-presenting cells respond differently to gut-derived probiotic bacteria but mediate similar strain-dependent NK and T cell activation. *FEMS Immunol Med Microbiol* 2007;51:535–46.
- [15] Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. *Annu Rev Immunol* 2003;21:685–711.
- [16] Moretta A. Natural killer cells and dendritic cells: rendezvous in abused tissues. *Nat Rev Immunol* 2002;2:957–64.
- [17] Cazzola M, Noschese P, Di Perna F. Value of adding a polyvalent mechanical bacterial lysate to therapy of COPD patients under regular treatment with salmeterol/fluticasone. *Ther Adv Respir Dis* 2009;3:59–63.