

ORIGINAL ARTICLE

Activation of T cells by dendritic cells exposed to a reference sensitizer: Towards a promising model to assess the allergenic potential of chemicals

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Background: Low molecular weight chemicals constitute one of the major causes of occupational allergies. European legislation on chemicals recommends limiting the use of *in vivo* models for assessing the sensitizing potential of chemicals, and encourages the development of integrated alternative methods. An *in vitro* mouse model of bone marrow-derived dendritic cells (BMDCs) that showed good accuracy (75%) and sensitivity (69%) has previously been developed to assess the sensitizing potential of chemicals.

Objective: To assess the ability of BMDCs to activate T cells (TCs) *in vitro*.

Methods: BMDCs pre-exposed to the reference sensitizer ammonium hexachloroplatinate (AHCP) were co-cultured with different subpopulations of TCs. TC activation was assessed by surface marker expression, proliferation, and cytokine release.

Results: The results showed significant activation of TCs co-cultured with dendritic cells pre-exposed to AHCP as evaluated by CD124 expression, proliferation, and cytokine secretion. Moreover, the response of TCs appeared to be Th2-oriented. Naive TCs were shown to be involved in this response, and the removal of regulatory TCs did not improve the cell response.

Conclusions: The BMDCs used in this previously developed model appear to have the ability to activate TCs, confirming that the BMDC model represents a reliable assay for assessing the sensitizing potential of chemicals.

KEYWORDS

chemical allergens, dendritic cells, *in vitro* assay, T cells

1 | INTRODUCTION

Occupational allergies constitute an important health issue that may be caused, in particular, by low molecular weight chemicals.^{1,2}

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Abbreviations: AHCP, ammonium hexachloroplatinate; BD, Becton Dickinson; BMDC, bone marrow-derived dendritic cell; CBA, cytometric bead array; CFSE, carboxyfluorescein diacetate succinimidyl ester; DC, dendritic cell; FBS, fetal bovine serum; HBSS, Hank's balanced salt solution; h-CLAT, human cell line activation test; IFN, interferon; IL, interleukin; IMDM, Iscove's modified Dulbecco's medium; LPS, lipopolysaccharide; RPMI, Roswell Park Memorial Institute; TC, T cell; Treg, regulatory T cell.

Exposure to these chemicals mainly induces contact hypersensitivity and respiratory sensitization, both of which can have important adverse health effects and can lead to detrimental socioeconomic consequences.^{3,4} Two pieces of legislation—(1) the 2008 amendment to the Regulation under the United Nations Globally Harmonized System for Classification, Labeling, and Packaging of substances and mixtures (Regulation [EC] No. 1272/2008)⁵ and (2) the European regulation of Registration, Evaluation, Authorization and Restriction of Chemical substances—recommend assessing the sensitizing potential of chemicals prior to their launch on the European market. In this context, *in chemico* and *in vitro* methods have been developed to

replace animal testing. Some of them have already been validated. These include the direct peptide reactivity assay (TG 442C⁶), an *in chemico* method; the KeratinoSens (TG 442D⁷), an *in vitro* method using a keratinocyte cell line; the human cell line activation test (h-CLAT; TG 442E⁸), which uses the human monocytic leukaemia cell line THP-1; the U-SENS (similar to the h-CLAT, but using the U937 cell line); and the interleukin (IL)-8 Luc assay, which uses the THP-1 cell line.⁸ Each of these tests deals with the first 3 of 4 key events in the sensitization process, namely, the covalent interaction with proteins, and keratinocyte and dendritic cell (DC) activation.⁹ Although some teams are currently working on *in vitro* T cell (TC) priming assays,^{9–11} none of the methods that address the activation of TCs, which is the final and decisive step in the sensitization process, have been validated to date. In the TC priming process, DCs play a central role through their ability to uptake, process and present haptenated self-proteins to TCs.^{11–13} Co-culture models involving different types of human DC and TC have already been described;^{14–17} however, they have a number of limitations, such as scarce supplies of fresh human cells, the handling of potentially infectious material, and wide interdonor variability. The use of mouse cells allows heterologous incompatibility to be avoided, and can lead to more homogeneous results.¹⁸

A DC activation model based on bone marrow-derived DCs (BMDCs) was previously developed in our laboratory, and showed encouraging results for a wide range of reference chemicals.^{19,20} In the present study, we aimed to assess the functionality of activated murine DCs by evaluating their ability to prime naive TCs and induce a proliferative response *in vitro*.

2 | METHODS

In order to evaluate the ability of DCs to prime TCs, the activated DCs were co-cultured with mouse splenic TCs. DC activation was carried out with lipopolysaccharide (LPS) and ammonium hexachloroplatinate (AHCP) (CAS no. 16919-58-7), a reference sensitizer.^{21–23} TC activation was evaluated by measuring cell proliferation, the expression of membrane markers, and cytokine secretion.^{4,24} To develop this model, the optimal conditions for cell culture were first determined. The most relevant activation parameters were then selected, and the roles played by the different subpopulations of TCs in the observed response were evaluated.

2.1 | Preparation of chemicals

The 2 substances (AHCP and LPS) were purchased from Sigma-Aldrich Chemical (Saint Quentin Fallavier, France), and were of the highest purity available. Chemicals were prepared in Hank's balanced salt solution (HBSS) (Invitrogen, Carlsbad, California). Fresh solutions were prepared for each experiment.

2.2 | Generation and treatment of immature DCs

Animal experiments were performed in line with ethical regulations on animal experimentation (European Directive 2010/63/EU) in an

animal facility accredited by the French Ministry of Agriculture (Accreditation D54-547-10). The present study was approved by the local ethics committee, and was registered as AE-2015-002.

BALB/c mouse BMDCs were generated with a method described previously.^{20,25} Cells were cultured in 150-mm Petri dishes, in Iscove's modified Dulbecco's medium (IMDM) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µM 2-mercaptoethanol (all from Life Technologies, Cergy-Pontoise, France), 10% heat-inactivated fetal bovine serum (FBS) (BioWest, Nuaille, France), and 50 ng/mL recombinant murine granulocyte-macrophage colony-stimulating factor (Preprotech, Rocky Hill, New Jersey). BMDCs were briefly trypsinized (0.05% trypsin/0.02% EDTA; Sigma-Aldrich Chemical) on day 7, and washed, counted and plated in 6-well plates in complete medium. Chemicals dissolved in HBSS were added over a period 24 hours. On day 8, the cells were briefly trypsinized (0.05% trypsin/0.02% EDTA; Sigma-Aldrich Chemical), centrifuged (370 g, 6 min), resuspended in fresh medium, and distributed at 90 000 cells per well in 96-well plates for co-culture with TCs. Control samples were treated with the vehicle HBSS for 24 hours. LPS was used as a BMDC-activating positive control at 100 ng/mL.

2.3 | Isolation of murine TCs and BMDC-TC co-culture

The spleens of female naive BALB/c mice aged 6–8 weeks were removed, sliced and pushed into a 70 µm cell strainer with a syringe plunger in Roswell Park Memorial Institute (RPMI) medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µM 2-mercaptoethanol, and 10% heat-inactivated FBS. TCs were enriched by selection with the mouse Pan T cell Isolation Kit II, the CD62L MicroBeads Kit, the CD25 MicroBead Kit, and the naive CD4⁺ and CD8a⁺ T Cell Isolation Kits (all from Miltenyi Biotec, Auburn, California), according to the manufacturer's instructions. Isolated TCs were added to the 96-well plates containing the pre-exposed DCs: 100 µL of the TC suspension in RPMI medium at 18×10^5 cells/mL was added to the wells containing 100 µL of the DC suspension in IMDM at 9×10^5 cells/mL (ratio: 1 DC/2 TCs). The plates were incubated at 37°C in 5% CO₂ for 12 to 72 hours.

Before the plating of TCs, carboxyfluorescein diacetate succinimidyl ester (CFSE) staining was performed in order to assess the proliferation of TCs.¹⁵ Fractionated TCs were labelled with CFSE for flow cytometric analysis of their proliferation. CFSE (Invitrogen, Molecular Probes, Eugene, Oregon) was added to cells to obtain a final concentration of 1.25 mM, and the cells were then incubated for 10 minutes at 37°C and washed twice in fresh medium before being distributed at 180 000 cells per well in the BMDCs containing 96-well plates for co-culture. Unlabelled cells were subjected to the same incubation and washing phases, but without the addition of CFSE. In order to avoid overlap between CFSE and monoclonal antibody fluorophores, some of the cells were left unstained for flow cytometric analysis of surface activation marker expression.

2.4 | Flow cytometry analysis and viability

CFSE-stained cells and non-CFSE-stained cells were analysed by flow cytometry. CFSE-stained cells were stained with PerCP-Cy5.5-conjugated anti-CD3 antibody (clone 145-2C11), BV786-conjugated anti-CD4 antibody (clone RM4-5), and PE-CF594-conjugated anti-CD8 antibody (clone 53-6.7). Non-CFSE-stained cells were stained with PerCP-Cy5.5-conjugated anti-CD3 antibody (clone 145-2C11), BV786-conjugated anti-CD4 antibody (clone RM4-5), PE-CF594-conjugated anti-CD8 antibody (clone 53-6.7), and PE-conjugated anti-CD11c antibody (clone HL3). To assess the activation of TCs, a number of different markers can be studied. Among these, we selected the hyaluronate receptor CD44, the very early activation antigen CD69, and the IL-4 receptor CD124.^{26–31} The antibodies used for the cytometry analysis included the following: BV605-conjugated anti-CD25 antibody (clone GL1), APC-Cy7-conjugated anti-CD44 antibody (clone IM7), APC-conjugated anti-CD69 antibody (clone H1.2F3) and BV421-conjugated anti-CD124 antibody (clone mL4R-M1) or isotype-matched control monoclonal antibodies. All antibodies were purchased from Pharmingen (BD Biosciences, le Pont de Claix, France). Surface staining was performed on fresh cells after co-culture, and the stained cells were analysed immediately on a Fortessa X20 flow cytometer with DIVA software (Becton Dickinson [BD], San Jose, California). BMDC population viability was assessed after 24 hours of exposure to chemicals by 7-aminoactinomycin D (7-AAD) staining (BD Biosciences, le Pont de Claix, France), and their activation was assessed with a PE-Cy7-conjugated anti-CD86 antibody (clone B7-2). The CD86 marker was analysed on the CD11c⁺ population (data not shown). After co-culture, debris and dead cells were eliminated from the analysis with a gate based on forward scatter and side scatter and by the use of LIVE/DEAD aqua fluorescent reactive dye (Life Technologies, Eugene, Oregon), respectively (Figure 1A). At least 20 000 cells were analysed for each sample.

FLOWJO v. 10.0.8 (FlowJo, Ashland, Oregon) was used for the analysis of FCS data files and the production of data illustrations. CD4⁺ and CD8⁺ gating was applied to the CD3⁺ population.

To evaluate TC activation, we used different membrane markers. CD44 is an adhesion molecule that is expressed at low levels on naive/resting TCs and upregulated on activated and memory TCs.²⁶ CD69 is a membrane-bound type II C-lectin receptor implicated in the homing and migration of activated lymphocytes.^{27,28} CD124 is the receptor of both IL-4 and IL-13 cytokines, whose (these?) are implicated in Th2 type differentiation, and its expression is upregulated on both CD4⁺ and CD8⁺ mature TCs.^{29,31} CD25, CD44, CD69 and CD124 markers were analysed on the CD3⁺, CD4⁺ or CD8⁺ populations, whereas the CD86 marker was analysed on the CD11c⁺ population. The percentage of positive cells was established by gating on the highly fluorescent cells (Figure 1A).

2.5 | Cytokine release

Interferon (IFN)- γ , IL-2, IL-4, IL-5, IL-9 and IL-13 production was used to explore activation of TCs.^{32–35} Supernatants were collected after 72 hours of co-culture, and stored at -80°C until analysis by cytometric bead array (CBA). BD CBA mouse IFN- γ , IL-2, IL-4, IL-5, IL-9

and IL-13 Flex Sets (BD Biosciences, San Jose, California) were used to quantify the amount of cytokine secreted in the supernatant, according to the manufacturer's instructions. The data were acquired with a BD FACSCanto II flow cytometry system (BD Biosciences), and the analyses were performed with BD FACS ARRAY v3.0 software (BD Biosciences).

2.6 | Statistical analysis

The results are presented as the mean of the percentage of positive cells $\pm$ standard error of the mean (SEM). Student's *t*-test was applied for testing of differences between 2 groups. One-way ANOVA followed by a multiple-range test (with Bonferroni correction) was performed for comparison of the effects of various concentrations of AHCP on TC activation with those of the vehicles. Differences were considered statistically significant for *P* values of $\leq .05$.

3 | RESULTS

3.1 | Characterization of the activation of TCs after 72 hours of co-culture with BMDCs pre-exposed to a chemical

We first characterized the activation of TCs in order to select the most relevant markers to use in our model. Under our conditions of stimulation, CD44, CD69 and CD124 were significantly upregulated on CD3⁺ cells co-cultured for 72 hours with BMDCs pre-exposed to LPS and AHCP (Figure 1B). The percentages of positive cells in the CD3⁺, CD4⁺ and CD8⁺ populations were higher with CD124 than with CD44 and CD69, so the CD124 surface marker was chosen to illustrate the following results.

The proliferation of TCs co-cultured for 72 hours with BMDCs pre-exposed to LPS and AHCP was significantly higher than that of TCs co-cultured with vehicle BMDCs. This increased proliferation was observed in the CD3⁺, CD4⁺ and CD8⁺ populations (Figure 1C).

IFN- γ , IL-4, IL-5, IL-9 and IL-13 concentrations in supernatants collected from co-cultures conducted with BMDCs pre-exposed to AHCP and LPS were significantly higher than in those pre-exposed to HBSS. A significant increase in IL-2 secretion was found in the presence of BMDCs pre-exposed to LPS, but not in the presence of those pre-exposed to AHCP (Figure 1D). To determine the contributions of the CD4⁺ and CD8⁺ TCs, the different patterns of marker upregulation and proliferation in the CD4⁺ and CD8⁺ subpopulations were examined. The results showed that the proportion of activated CD8⁺ TCs was higher than the proportion of activated CD4⁺ TCs (Figures 1B, 2, and 3).

3.2 | Influence of co-culture time on CD124 expression and proliferation of TCs

We investigated the influence of co-culture time on both the proliferation of TCs and their expression of CD124. The CD124 marker was strongly expressed on CD4⁺ and CD8⁺ TCs after 12, 36 and 72 hours of co-culture with BMDCs pre-exposed to LPS. CD124 showed significantly higher expression on both types of TC after 36 and

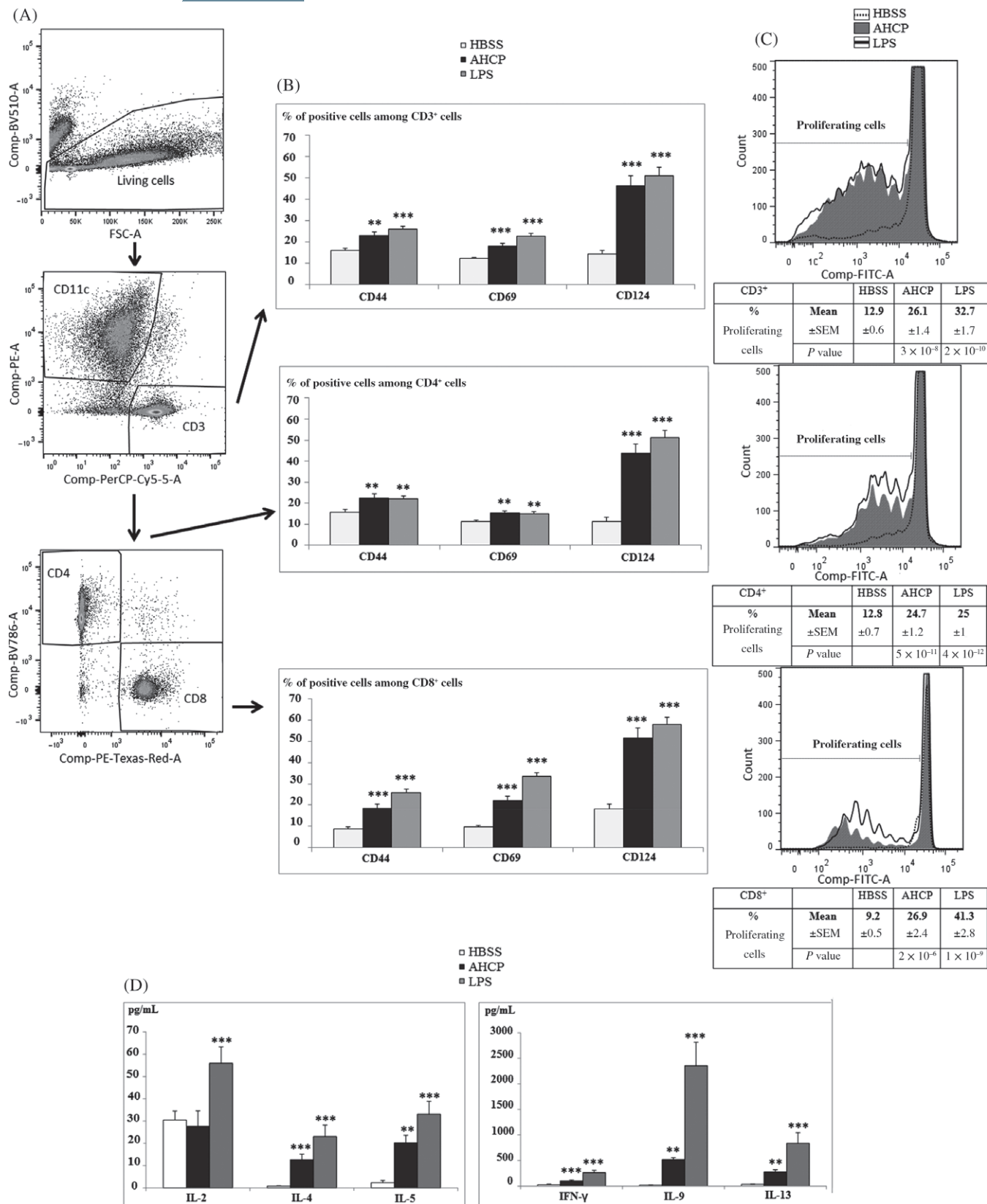


FIGURE 1 Phenotypic characterization of T cell (TC) activation after 72 hours of co-culture with bone marrow-derived dendritic cells (BMDCs) pre-exposed to chemicals. TCs were cultured for 72 hours with BMDCs pre-exposed to 100 μ M ammonium hexachloroplatinate (AHCP), 100 ng/mL lipopolysaccharide (LPS), or the Hanks balanced salt solution (HBSS) vehicle control, and then stained with specific antibodies and analysed by flow cytometry. **A**, Data were processed with FLOWJO software with gating on live cells and CD3⁺ cells. Within the CD3⁺ population, the CD4⁺ and CD8⁺ populations were gated. **B**, CD44, CD69 and CD124 activation markers were studied in CD3⁺, CD4⁺ or CD8⁺ populations. **C**, After carboxyfluorescein diacetate succinimidyl ester staining, proliferation was assessed in the CD3⁺ TC population and in its CD4⁺ and CD8⁺ subpopulations. The histograms show an example of each proliferating cell population. The table under each figure shows the mean $\pm$ SEM of the percentage of proliferating cells in each population, and the *P* values for each chemical. **D**, Cytokine secretion was measured in the supernatant after 72 hours of co-culture. Assays were performed 7 times with AHCP and 9 times with LPS. Data are mean $\pm$ SEM. **P* < .05; ***P* < .01; ****P* < .001. IFN, interferon; IL, interleukin

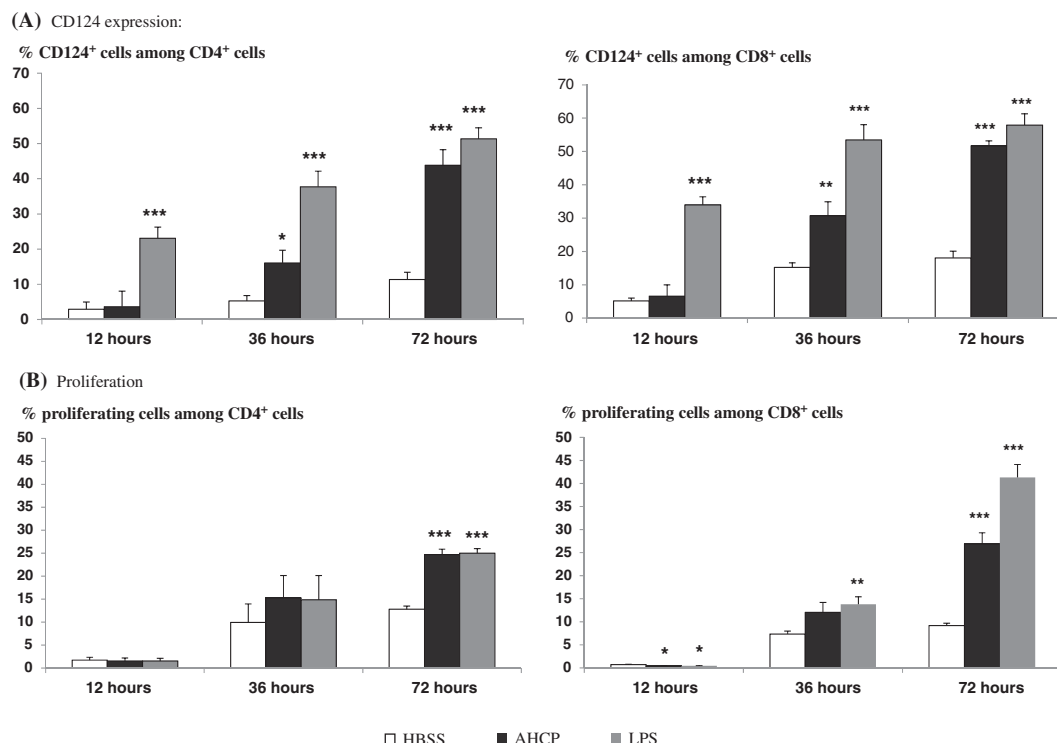


FIGURE 2 Phenotypic characterization of T cell (TC) activation after various durations of co-culture with bone marrow-derived dendritic cells (BMDCs) pre-exposed to chemicals: CD124 expression (A) and carboxyfluorescein diacetate succinimidyl ester-stained proliferating TCs (B) were studied by flow cytometry after 12, 36 and 72 hours of culture with BMDCs pre-exposed to 100 μ M ammonium hexachloroplatinate (AHCP), 100 ng/mL lipopolysaccharide (LPS), or the Hank's balanced salt solution (HBSS) vehicle control. Assays were performed 7 times with AHCP and 9 times with LPS at 72 hours, and twice with both AHCP and LPS at 12 and 36 hours. Data are mean $\pm$ SEM. * P < .05; ** P < .01; *** P < .001

72 hours of co-culture with BMDCs pre-exposed to AHCP than after co-culture with BMDCs pre-exposed to HBSS. The maximum significance of CD124 expression, in both the CD4⁺ population and the CD8⁺ population, was observed after 72 hours of co-culture (Figure 2A).

At 36 hours, significant proliferation could only be observed for CD8⁺ cells co-cultured with BMDCs pre-exposed to LPS. At 72 hours, significant proliferation was observed for both CD4⁺ and CD8⁺ cells co-cultured with DCs pre-exposed to LPS or AHCP (Figure 2).

Owing to high mortality rates at 96 and 120 hours of co-culture (from 35% to 50% mortality; data not shown), the results were not considered at these 2 time-points of co-culture.

3.3 | Effects on TC activation of exposure of BMDCs to various concentrations of AHCP

The effects of different concentrations of AHCP (0, 12.5, 50, 75, 100, 150 and 200 μ M) were studied. The results showed that TC proliferation increased significantly at concentrations of AHCP of 50 μ M and above, in both the CD4⁺ population and the CD8⁺ population. The maximum rates of cell proliferation were reached at 100 μ M for the CD4⁺ population and at 150 μ M for the CD8⁺ population. BMDC viability was below the threshold of 80% after 24 hours of exposure to 150 and 200 μ M AHCP (Figure 3).

3.4 | Responses of different TC populations after co-culture with BMDC pre-exposed to AHCP

We next investigated the contribution of naive and memory TCs to the observed response in this model. To select the different subpopulations of TCs, we used enrichment kits to select CD62L⁺ and CD44⁺ TCs. CD62L is a marker used to characterize naive TCs,³⁶ whereas CD44 is used to characterize memory TCs.³⁷ Whereas both CD62L⁺ and CD62L⁺ cells were activated after 72 hours of co-culture with BMDCs pre-exposed to LPS, AHCP stimulation of BMDCs led to a mainly CD62L⁺ TC response (Figure 4). Proliferation and CD124 expression were compared between pan-TCs and CD4⁺ CD44⁺ cells in co-culture with BMDCs pre-exposed to AHCP and LPS. The proliferation response and CD124 expression were higher with AHCP than with HBSS in both pan-TCs and CD4⁺ CD44⁺ cells. Both increases were similar between pan-TCs and CD4⁺ CD44⁺ cells (Table 1). The expression of CD124 on CD4⁺ CD44⁺ cells was not significantly higher with LPS than with HBSS, but CD124 expression was significantly higher on pan-TCs. When CD8⁺ CD44⁺ cells were cultured alone with BMDCs pre-exposed to AHCP or LPS, neither activation nor surface marker expression was observed (Table 1).

To assess the involvement of regulatory T cells (Tregs), we used a kit that selects CD25⁺ Tregs.¹⁶ After enrichment of the whole TC population in CD25⁺ cells, proliferation and CD124 marker expression were assessed. Table 1 shows that, with CD25⁺ enriched cells,

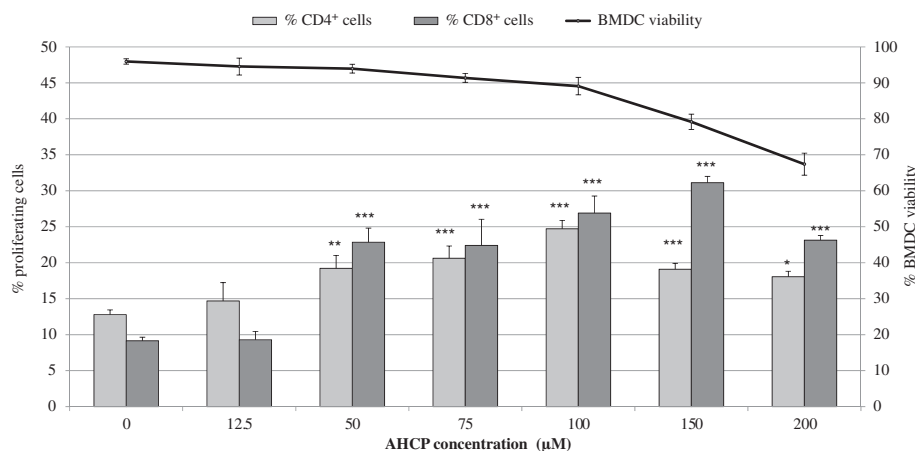


FIGURE 3 Effects of bone marrow-derived dendritic cell (BMDC) exposure to various concentrations of ammonium hexachloroplatinate (AHCP) on T cell activation. The percentages of live BMDCs after 24 hours of exposure to increasing concentrations of AHCP (0 [Hank's balanced salt solution], 12.5, 50, 75, 100, 150 and 200 μM) are shown by the black line. Data are mean $\pm$ SEM. *P < .05; **P < .01; ***P < .001

there was a decrease in the significance level, both in the proliferating and in the CD124⁺ cells. The results were not significant for proliferation of CD25⁺ cells with either AHCP or LPS, or for CD124 expression with AHCP.

4 | DISCUSSION

In this study, we assessed the ability of DCs pre-exposed to a well-known reference sensitizer, AHCP,^{19,21,23} to activate TCs. For this purpose, DCs were co-cultured with TCs, and TC activation was evaluated through the expression of membrane markers on the TCs, cell proliferation, and cytokine secretion. First, we investigated the most relevant parameters for evaluating the activation of TCs co-cultured with DCs pre-exposed to LPS or AHCP. To evaluate the activation of TCs, 3 different membrane markers were used: CD44, CD69, and CD124. The results showed that all 3 activation markers were upregulated with both LPS and AHCP (Figure 1), showing that activated DCs were capable of inducing their expression and activating TCs. Lymphocyte proliferation was also used to characterize the response to allergens.⁴ Co-culture with DCs pre-exposed to AHCP led to

significant proliferation of TCs in our model, confirming the activation of TCs (Figure 1).

There is a significant difference in the proportions of CD4⁺ (70%) and CD8⁺ (25%) cells (data not shown) among the CD3⁺ TCs in mouse spleen. It is therefore important to discriminate between these 2 populations when analysing our results to ensure that the activation of CD8⁺ cells is not missed as a result of the higher proportion of CD4⁺ cells. Massive expansion of CD8⁺ TCs in response to antigens has already been described in another study.³⁸ In agreement with our results, the activation of the CD8⁺ TC population triggered by AHCP had previously been related to metal allergy and hapten-induced contact hypersensitivity.^{39–41}

The CD124 marker was selected as the most relevant for assessing TC activation in this model, as it showed the highest increases with AHCP and LPS as compared with HBSS. CD124 membrane marker expression has already been described in the Th2-type immune response,^{33,42,43} and AHCP has been reported to induce a Th2-type immune response after *in vivo* exposure.^{4,44–46} The following hypothesis can be suggested to explain the expression of CD124 induced by AHCP: DCs activated by their exposure to AHCP activate TCs, inducing IL-4 secretion, as shown in Figure 1D. The secreted IL-

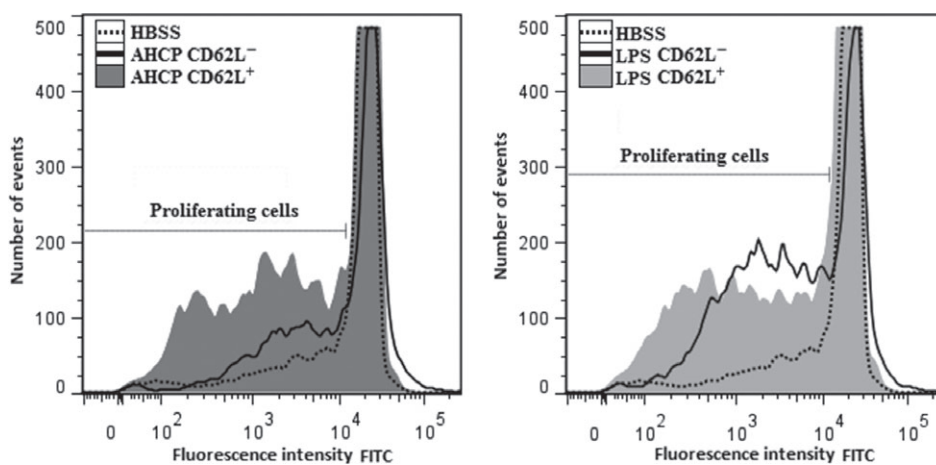


FIGURE 4 Activation of the CD62L⁺ and CD62L⁻ T cell populations by bone marrow-derived dendritic cells (BMDC) pre-exposed to lipopolysaccharide (LPS) and ammonium hexachloroplatinate (AHCP): CD3⁺ CD62L⁺ or CD3⁺ CD62L⁻ enriched cell populations stained with carboxyfluorescein diacetate succinimidyl ester were co-cultured for 72 hours with BMDCs pre-exposed to 100 μM AHCP, 100 ng/mL LPS, or the Hank's balanced salt solution (HBSS) vehicle control. The histograms show an example of each proliferating population under the different exposure conditions. FITC, fluorescein isothiocyanate

TABLE 1 Activation of different T cell (TC) populations obtained by pan T cell selection, CD25 depletion and CD4⁺ CD44⁻ / CD8⁺ CD44⁻ selection: CD25⁻, CD4⁺ CD44⁻ or CD8⁺ CD44⁻ TCs were co-cultured for 72 hours with bone marrow-derived dendritic cells pre-exposed to 100 μ M ammonium hexachloroplatinate (AHCP), 100 ng/mL lipopolysaccharide (LPS), or the Hank's balanced salt solution (HBSS) vehicle control, and the results were then compared with those of assays performed on the pan-TC population

		Pan-TCs		CD25 ⁻		CD4 ⁺ CD44 ⁻		CD8 ⁺ CD44 ⁻	
		% proliferating cells	% CD124 ⁺ cells	% proliferating cells	% CD124 ⁺ cells	% proliferating cells	% CD124 ⁺ cells	% proliferating cells	% CD124 ⁺ cells
HBSS	Mean $\pm$ SEM	12.9 $\pm$ 0.6	14.3 $\pm$ 1.8	20 $\pm$ 6.8	14.5 $\pm$ 4	7.4 $\pm$ 1.5	23.1 $\pm$ 7.2	8.3 $\pm$ 1.3	3.9 $\pm$ 0.8
AHCP	Mean $\pm$ SEM	26.1 $\pm$ 1.4	46.3 $\pm$ 4.5	28.2 $\pm$ 5.4	33.3 $\pm$ 7	21.5 $\pm$ 3	55.4 $\pm$ 5.1	6 $\pm$ 1	4.1 $\pm$ 0.5
	P value	< .0001	< .0001	NS	NS	.0004	.004	NS	NS
LPS	Mean $\pm$ SEM	32.7 $\pm$ 1.7	51 $\pm$ 3.8	35 $\pm$ 4.9	48.9 $\pm$ 7.3	15.8 $\pm$ 2.8	38.7 $\pm$ 5.8	9.5 $\pm$ 1.4	8.6 $\pm$ 3.1
	P value	< .0001	< .0001	NS	.006	.008	NS	NS	NS

NS, not significant; SEM, standard error of the mean.

The table shows the mean percentages of proliferating cells and of the CD124⁺ population $\pm$ SEM, and the P values for each culture condition. Assays were performed 7 times with AHCP and 9 times with LPS in pan-TCs, and 3 times in CD25⁻, CD4⁺ CD44⁻ and CD8⁺ CD44⁻ cells.

4 binds to the IL-4 receptor α chain (CD124),⁴⁷ and, through the JAK-STAT signalling pathway,⁴⁸ leads to transcription of the IL-4R gene and upregulation of CD124.⁴⁹ It is therefore possible that the expression of CD124 was linked to the Th2-type response, and that another sensitizer would not have induced this membrane marker expression. Even though the CD124 marker was efficient with AHCP (Figure 1B), it is possible that the upregulation of CD124 is less pronounced after exposure to a sensitizing agent known to induce a Th1 response, such as dinitrochlorobenzene. Thus, although we will test other reference chemical sensitizers with this model, we need to continue to analyse CD44, CD69 and CD124 markers when testing other known sensitizers with our model, especially known Th1-inducing chemicals. As far as cytokines were concerned, we observed increased secretion of Th2-type cytokines such as IL-4, IL-5 and IL-13 in the supernatant of cells exposed to AHCP. In contrast, secretion of the Th1-type cytokine IL-2 was not modified by AHCP but was increased by LPS exposure. Together with the fact that the CD124 marker is also involved in Th2 differentiation, the data seem to be consistent with a Th2-oriented response. The Th2 orientation of the BALB/c strain could partly explain the Th2-oriented response observed. However, as shown by Nishino et al,⁵⁰ Th1-oriented strains such as C57/BL6N are susceptible to producing Th2-type cytokines in response to exposure to respiratory allergens. The specificity of the Th2-type response will be investigated in the future with assays on C57BL/6 mouse cells and other types of chemical allergen.

We then evaluated the concentration-dependent effect of AHCP on TC activation by examining increasing AHCP concentrations, ranging from 0 to 200 μ M. These results showed a proportional effect of the concentration of AHCP on the activation of TCs by pre-exposed DCs. The maximum concentration-dependent effect depended on the subpopulation, that is, CD4⁺ or CD8⁺, which illustrates the discrepancy between the 2 subpopulations of TCs in terms of their response to pre-exposed DCs. After determining the most relevant activation markers for TCs, and ensuring that a concentration-related AHCP response had been observed, we determined the optimum co-culture time for detecting TC activation. As the highest expression of CD124 and proliferation of both CD4⁺ and CD8⁺ TCs were observed after 72 hours of co-culture, and as the mortality rate of TCs increases beyond this duration of co-culture, the 72-hour time-point

was selected. As compared with data obtained with other DC-LT co-culture models, 72 hours seems to be a relatively short time in which to observe a significant response.^{14,15,17}

TC populations are heterogeneous, and this could have an impact on the results. To assess the involvement of naive and memory TCs in the observed response, CD62L⁺ and CD62L⁻ TC populations were separated. It was shown that CD62L⁺ and CD62L⁻ TCs proliferated when DCs were exposed to LPS, whereas only CD62L⁺ naive TCs proliferated when DCs were exposed to AHCP. These data show that the response of TCs to AHCP is attributable to naive TCs, and not to memory TCs. To further explore the naive memory cell response, we assessed both CD4⁺ CD44⁻ and CD8⁺ CD44⁻ (CD44⁻ cells are the non-stimulated TCs²⁶) populations isolated by magnetic separation (Table 1). The results showed, that even in the absence of memory and CD8⁺ TCs, the proliferation of CD4⁺ cells increased significantly, as did CD124 expression, with AHCP as compared with the vehicle control. In contrast, with LPS, no significant increase in CD124 expression was observed, which supports the minor involvement of memory TCs in the AHCP response. These results are in agreement with other studies showing that DCs were unable to activate CD8⁺ CD44⁻ TCs in the absence of CD4⁺ TCs.^{51,52}

The presence of Tregs may reduce the cell response to stimulation, leading to false-negative results.¹⁶ It has been shown in previous studies that Tregs can prevent *in vitro* activation of TCs in response to moderate/weak allergens, and that the depletion of human CD25⁺ peripheral blood lymphocytes may be useful to significantly increase specific TC activation.¹⁶ We therefore depleted the CD25⁺ cells in the pan-TC population in order to verify whether this could increase the TC response to activated DCs. The results in Table 1 show that there was no increase in the proliferation or the percentage of CD124⁺ cells in the CD25⁻ population. In contrast, the higher background level in the proliferating cells and the higher variability in the response of TCs led to a decrease in the significance level when the CD25⁺ cells were removed from the TC population. We therefore assumed that, with this model, CD25⁺ cell depletion is not required to observe an appropriate response of TCs. Tregs have a tendency to lower the TC response.²⁴ Thus, the discrepancy between the literature data and the data provided by this model might be explained by

the use of murine cells instead of human cells, or by a shorter co-culture time than that used in other models.¹⁶

To conclude, we have developed a murine co-culture model that allows us to assess the ability of DCs exposed to AHCP—a reference sensitizer—to induce activation of TCs. These preliminary results are promising for the development of an easy-to-use in vitro model. This model has the advantage of not requiring the use of live animals, and, moreover, the BMDCs and TCs used in this model are both easy to obtain and homogeneous. Furthermore, the 72 hours of co-culture required to observe the TC response is shorter than in other models based on the same cell types.^{14,15,17} Our model, which explores the response of TCs, might also allow discrimination of Th1 and Th2 responses, which would be an interesting information in the development of alternatives to live animal experimentation. There is therefore a need to assess other reference chemical sensitizers, such as strong and weak sensitizers, especially those known to induce either Th1 or Th2 responses, as well as non-sensitizers, in order to use this co-culture model as a predictive test for assessing the allergenic potential of chemicals.

Conflict of interest

The authors declare no potential conflict of interests.

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