



Differentiation of BMDC with CpG and Gm-CSF, But Not CpG and Flt3L, Imprints CCR9 Expression on Co-Cultured T Cells

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Abstract

Dendritic cells pulsed with *Burkholderia pseudomallei* were used to activate naïve T-cells *in vitro*. Murine bone-marrow- derived dendritic cells (BMDC), cultured with CpG and GM-CSF or CpG and Flt3L *ex vivo*, were pulsed with killed *B. pseudomallei* or the Burkholderia protein Lolc, and co-cultured with naïve T-cells. GM-CSF- derived BMDC pulsed with *B. pseudomallei* and CpG were activated, with CD40, CD80, CD86 and MHCII expression. Lolc, in the absence of CpG, was not immunostimulatory. GM-CSF-cultured BMDC, pulsed with *B. pseudomallei* and CpG, expressed CCR7 and imprinted CCR9 on co-cultured naïve T-cells, unlike BMDC cultured with Flt3L and CpG. GM-CSF-cultured, but not Flt3L-cultured BMDC pulsed with *B. pseudomallei* and CpG, had significantly up-regulated ALDH activity in the retinoic acid pathway. CCR9 expression on T-cells activated by GM-CSF-cultured BMDC pulsed with *B. pseudomallei* and CpG, was undiminished by pre-exposing BMDC to lung, spleen or skin tissue factors.

Previously, we have observed that GM-CSF and CpG- cultured *B. pseudomallei*-pulsed BMDC, induced protective efficacy against *B. pseudomallei* challenge on adoptive transfer into naïve mice. The current data suggest that the protective mechanisms induced *in vivo*, are associated with the influence of GM-CSF and CpG on DC, to up regulate ALDH and to imprint *B. pseudomallei*-specificity and migratory potential on co-cultured T cells.

Keywords: CCR9; T-cells; BMDC; GM-CSF; FLT3L; CpG

Introduction

Burkholderia pseudomallei is an aerobic, asporogenic, Gram-negative bacillus and the etiological agent of melioidosis, a disease endemic to Southeast Asia, northern Australia and other tropical and subtropical regions [1]. The natural habitat of *B. pseudomallei* is still or stagnant water, rice paddies and moist tropical soil; the bacterium can infect individuals through cuts and abrasions or via inhalation of aerosols. The majority of reported melioidosis cases are in Thailand and northern Australia where they form the third most frequent cause of death from infectious disease [2].

Melioidosis can manifest as a chronic disease establishing latency within an intracellular niche, through to an acute disease with overwhelming septicemia [3]. Clinical symptoms include a flu-like illness with abscess formation in any of a variety of locations including the lungs, liver and spleen being characteristic of the disease [4]. *B. pseudomallei* infections are difficult to treat due to the absence of a licensed vaccine for prophylaxis [5], resistance of the bacteria to multiple antibiotics, the ability to form biofilms and the establishment of a chronic, intracellular infection.

Dendritic cells (DCs) provide an interface between innate and adaptive immunity [6,7]. They are a heterogeneous group of antigen presenting cells (APCs) [8-12] and are located in tissues that represent pathogen entry routes, including the skin, lungs and gastrointestinal tract. DCs have been classified based on their function, location and/or morphology, separating monocyte- and macrophage- derived DCs. Thus DCs can be divided into classical DC1 (cDC1), cDC2 and plasmacytoid DC (pDCs) populations, defined in part by the expression of transcription factors [10,13] and cell surface markers.

In response to danger signals from pathogens, peripheral DCs mature with the up regulation of cell surface molecules including CD40, CD80, CD86 and MHCII and also CCR7, which enables their migration along CCL19 and CCL21 gradients to lymph nodes [11,13-15]. Within lymph nodes, DCs

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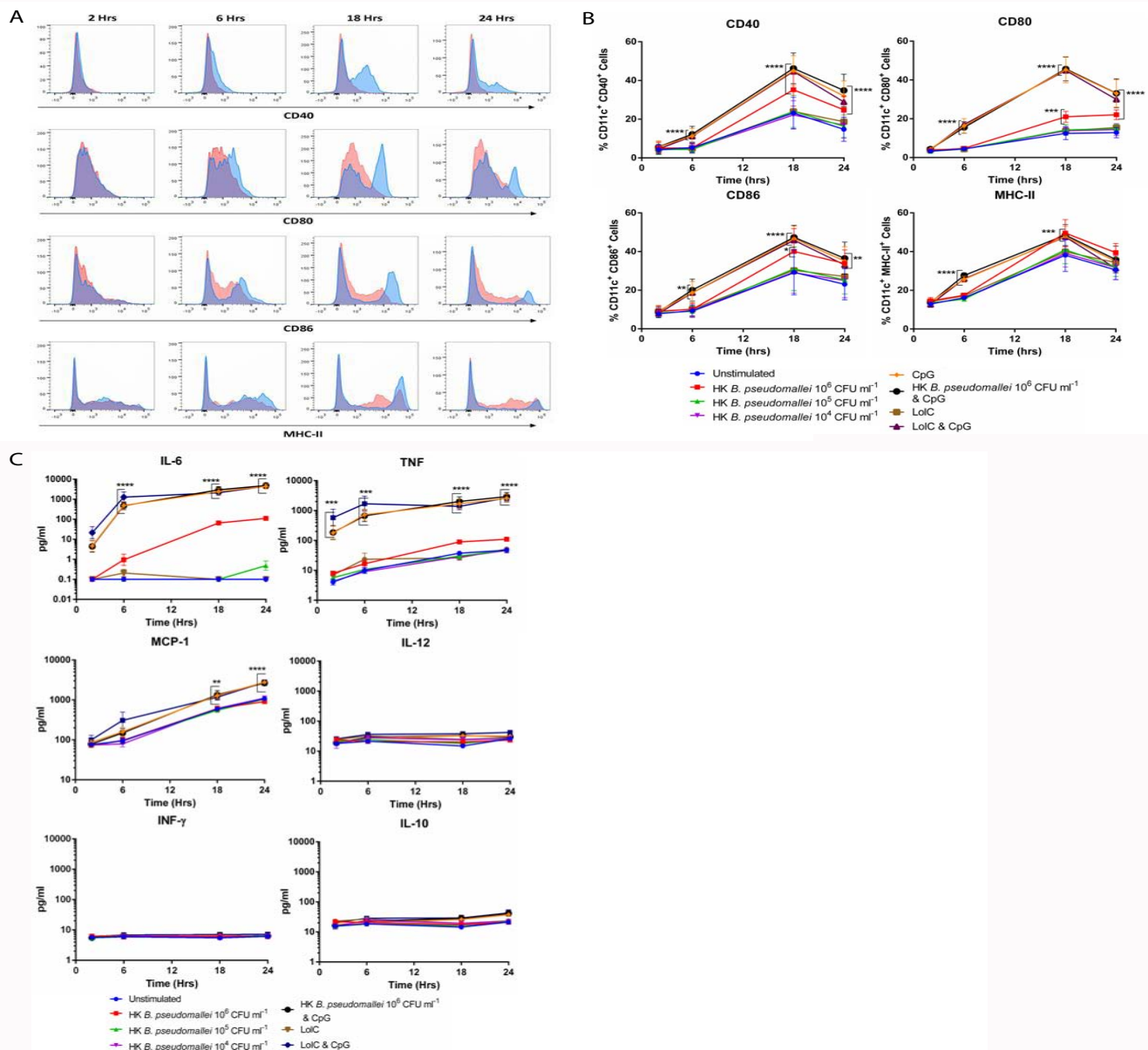


Figure 1: BMDCs increase expression of T cell costimulatory molecules in response to HK *B. pseudomallei* + CpG. Murine BMDCs were pulsed for either 2, 6, 18 or 24 h. with HK *B. pseudomallei* or LolC (10 µg ml⁻¹) ± CpG (6 µg ml⁻¹), with CpG alone, or were unstimulated. BMDCs were then stained for CD11c and costimulatory molecules CD40, CD80, CD86, and MHC-II. Expression levels were quantified by flow cytometry. (A) Representative fluorescence intensity histogram overlays of cells stimulated with HK *B. pseudomallei* 10⁶ CFU ml⁻¹ + CpG (blue histogram) and unstimulated cells (red histogram). (B) Aggregated data of three independent experiments represented as mean ± SEM. (C) Cytokine secretion profiles in activated BMDC. Concentration of cytokine within the BMDC culture supernatant was quantified using BD Mouse Inflammation Cytokine Bead Array. Data displayed represent mean ± SEM of three independent experiments. Statistical significance compared to unstimulated cells was determined by 2-way ANOVA. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001 and **** p ≤ 0.0001.

activate T cells by presenting peptides to them in association with MHC molecules, conditioning them through cytokine production and imprinting tissue-homing on them. The phenomenon of DC-mediated migration of T cells has been best characterized in tissues such as the skin, lungs and gut [15] in which localised vitamin metabolism provides tissue-specific conditioning factors [16]. T cells activated by DCs located in gut-associated lymphoid tissue (GALT) are able to induce expression of CCR9 and α4β7 on effector T cells [17], with their ligands, MAdCAM-1 and CCL25 respectively, expressed selectively by intestinal tissue, predominantly in the ileum and colon [18]. This conditioning is dependent on the production of the vitamin A metabolite retinoic acid (RA) by DCs. The *in vitro* culture of DCs in the presence of RA is able to induce the expression

of CCR9 and α4β7, resulting in gut-homing T cells [16]. Similarly, skin-resident DCs produce the active form of vitamin D, 1,25-dihydroxycholecalciferol (1,25(OH)₂D) which imprints T cells to express the chemokine receptor CCR10. The CCR10 ligand, CCL27 is selectively expressed by epidermal keratinocytes [19], causing effector T cells to selectively migrate to sites of skin inflammation. The expression of the chemokine receptor CCR4 has recently been suggested to contribute to the tropism of T-cells for the lung [20].

However infection by respiratory pathogens can result in accumulation of T cells in intestinal tissue [21], whilst lung DCs can induce the RA-dependent expression of CCR9 and α4β7 on T cells, unlike splenic or skin DCs [22]. Thus T cell imprinting may be more

complex than the current model suggests and lends credence to the concept of a common mucosal immune system, a hypothesis first proposed in 1978 [23].

The ability of DCs to induce specific immunity to infection has been exploited in a range of infection models. We and others have shown that murine BMDC can be cultured and activated appropriately *ex vivo* to induce specific cellular immunity when adoptively transferred into naive mice [24]. This process has been shown to induce protective efficacy against both *Bacillus anthracis* and *B. pseudomallei* in murine models [25,26]. Furthermore, active immunization against *Yersinia pestis* and HIV has been achieved in murine models by targeting DCs with antibodies to surface receptors such as CD205, fused to an antigen of interest [27,28]. Active immunization by targeting DC *in situ* is likely to replicate more closely the *in vivo* development of DCs, their activation, migration and imprinting of T-cells for tissue-specific migration [28]. In this study, we have investigated the influence of culture and activation conditions for BMDC on their ability to stimulate effector T cells optimally, so that when exploited *in vivo*, these activated BMDCs could imprint appropriate tissue-specific migratory potential to prevent infection with *B. pseudomallei*.

Results

BMDC culture and phenotyping

BMDCs derived by *ex vivo* culture with GM-CSF may exhibit a phenotype consistent with one or more subsets, since they are not subjected to the complex developmental pathways that common dendritic cell precursors (CDPs) experience *in vivo*. Here, GM-CSF was added to the media for BMDC culture and assay *in vitro*, unless otherwise stated. To determine if the GM-CSF-cultured BMDCs represented a homogeneous subset, they were selected for CD11c and then stained for the cell surface markers CD103, B220 and CD11b. Sixty-five percent of GM-CSF cultured BMDCs were CD11c⁺ and were identified to be predominantly (94%) CD11b⁺ CD103⁻ B220⁻, classifying them as cDC2-like cells.

BMDC can be activated by pulsing with heat-killed *B. pseudomallei* ± CpG

Evidence from earlier work revealed that the inclusion of the immunostimulant CpG in the activating medium used to pulse GM-CSF-cultured BMDC with heat-killed bacteria is essential, although the mechanism of activation was unclear. Here, GM-CSF-cultured BMDCs pulsed with heat-killed *B. pseudomallei* (10⁶ CFU) together with CpG displayed a time-dependent expression of co-stimulatory molecules CD40, CD80, CD86 and MHCII (Figure 1A). BMDC pulsed with 10⁶ CFU heat-killed *B. pseudomallei* had significantly enhanced expression of co-stimulatory molecules compared with unstimulated BMDC, but the addition of CpG to the activating medium significantly enhanced expression (Figure 1B). The expression of CD40, CD80, CD86 or MHC-II in BMDCs pulsed with heat-killed *B. pseudomallei* at concentrations below 10⁶CFU ml⁻¹, was not significantly different from unstimulated control BMDCs (Figure 1B). LolC alone did not significantly enhance the expression of any co-stimulatory molecule at any time point, compared to unstimulated cells, whereas the addition of CpG to LolC had the effect of increasing expression to the level of CpG alone.

All BMDC activating conditions which incorporated CpG, resulted in a significantly increased secretion of IL-6 and TNFα following 6, 18 and 24 hours pulsing, compared to unstimulated

BMDCs (Figure 1C). Similarly, BMDC activation in the presence of CpG significantly enhanced MCP-1 secretion at 18 and 24 hours. However, in the absence of CpG, there was no significant increase in concentrations of IL-6, TNFα or MCP-1, compared to unstimulated cells. In all conditions, there was no significant increase in the production of IL-12, IFN-γ or IL-10, compared to unstimulated control BMDCs. Taken together, these data suggest that Lolc is not immunostimulatory for BMDCs, that heat-killed *B. pseudomallei* is activating only above 10⁶ CFU ml⁻¹ and that CpG is a potent activator of BMDCs. Based on these time-course data, BMDCs were pulsed with heat-killed *B. pseudomallei* at 10⁶cfu ml⁻¹ ± CpG for 18 hours in all subsequent experiments.

Migration markers on pulsed BMDC

The interaction of specifically- activated BMDC with T-cells *in vivo* requires BMDC migration to lymph nodes. Here, the migratory potential of *in vitro*- activated GM-CSF-derived BMDC was assessed by their expression of CCR7. BMDCs were either unstimulated, or pulsed with heat killed *B. pseudomallei* (10⁶ CFU ml⁻¹) with or without CpG, or pulsed with CpG alone, prior to the detection of CD11c and CCR7 by flow cytometry. CCR7 expression was significantly enhanced on BMDCs activated with HK *B. pseudomallei* with CpG, compared to unstimulated cells (Figure 2); however, CCR7 expression was enhanced to the same extent by CpG alone, suggesting that this effect was independent of heat killed *B. pseudomallei* and that CpG was directly activating GM-CSF cultured BMDC to induce CCR7 expression.

Activation of BMDC prior to co-culture with T-cells

Evidence from our previous work showed that immunization of naïve mice with BMDC which had been cultured *ex vivo* with GM-CSF and pulsed with heat-killed *B. pseudomallei* and CpG prior to administration, induced *B. pseudomallei*-specific T-cell immunity

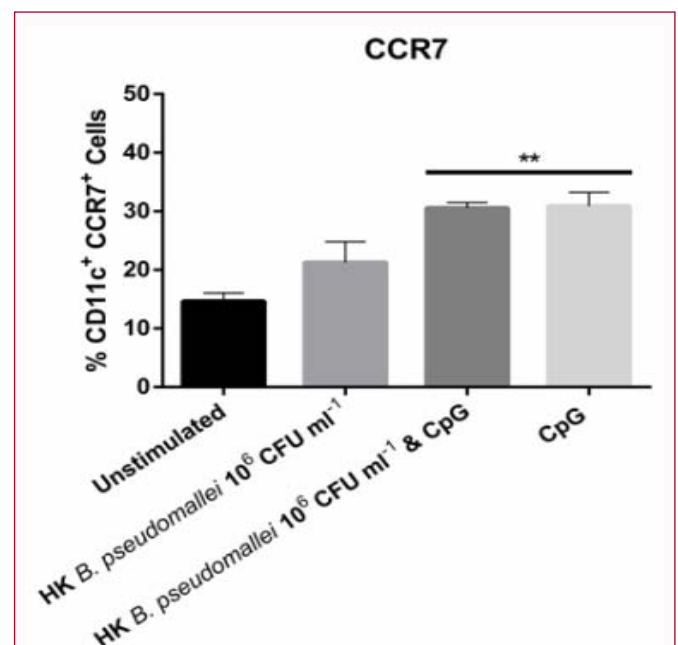


Figure 2: Activated BMDC have enhanced CCR7 expression. 'BMDCs' were pulsed for 18 h. with HK *B. pseudomallei* 10⁶ CFU ml⁻¹ ± CpG (6 µg/ml⁻¹), with CpG alone or were unstimulated. Cells were then analysed for the expression of CD11c and CCR7. Data represent mean ± SEM of three independent experiments. Statistical significance compared to unstimulated cells was determined via one-way ANOVA. ** p ≤ 0.01.

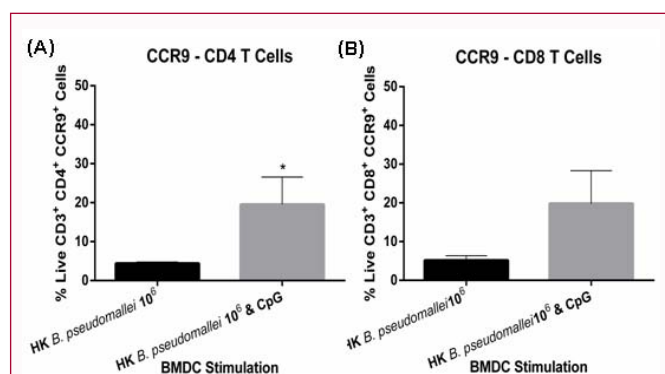


Figure 3: GM-CSF-cultured BMDCs activated with CpG induce T cells *in vitro* to express increased levels of gut-associated homing molecule CCR9. BMDCs were stimulated with HK *B. pseudomallei* 10^6 CFU ml^{-1} \pm CpG (6 $\mu\text{g ml}^{-1}$) for 18 h. Subsequently, BMDCs were co-cultured for 5 days at 1:1 ratio with naïve murine CD4+ or CD8+ T cells purified from murine spleen. The expression of T cell markers CD3, CD4, CD8 and CCR9 was quantified by flow cytometry. Data represent the mean \pm SEM of three independent experiments. Statistical significance was determined using a paired t test. * $p \leq 0.05$.

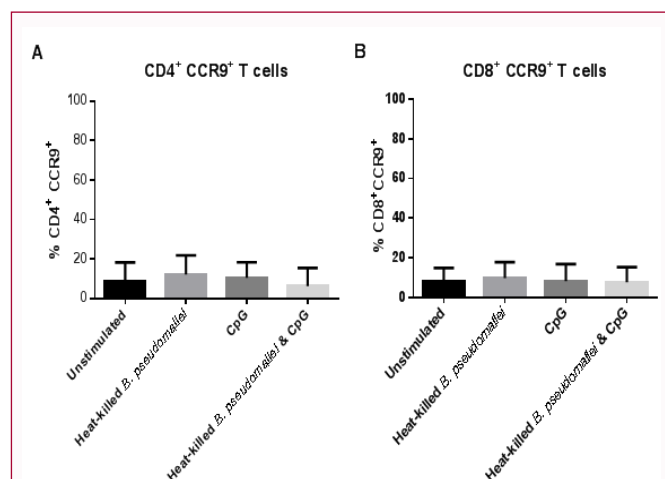


Figure 4: FLT3-cultured BMDCs, activated with CpG do not induce T cells *in vitro* to express CCR9. BMDCs were stimulated with HK *B. pseudomallei* 10^6 CFU ml^{-1} \pm CpG (6 $\mu\text{g ml}^{-1}$) for 18 h. Subsequently, BMDCs were co-cultured for 5 days at 1:1 ratio with naïve murine CD4+ or CD8+ T cells purified from murine spleen. The expression of T cell markers CD3, CD4, CD8 and CCR9 was quantified by flow cytometry. Data represent the mean \pm SEM of three independent experiments. Statistical significance was determined using a paired t test.

and was fully protective against challenge with *B. pseudomallei* [25]. It was of interest therefore to determine the influence of this preparative protocol on the expression of activation markers by BMDCs and their ability to imprint migration markers on co-cultured T-cells *in vitro*.

BMDCs were cultured as before for 6 days in GM-CSF-supplemented medium, prior to activation by pulsing with heat killed *B. pseudomallei* (10^6 ml^{-1}) \pm CpG for 18h. Subsequently, activated BMDC were washed and cultured with naïve CD4+ or CD8+ murine T-cells for 5 days, prior to the determination of T-cell activation. Co-cultured T-cells, whether CD4+ or CD8+, consistently expressed CCR9 which was significantly enhanced by the addition of CpG to the pulsing medium (Figure 3). However, T-cells which were co-cultured with BMDC which had been cultured in FLT3L-supplemented medium, rather than GM-CSF and activated with heat killed *B. pseudomallei* (10^6 ml^{-1}) \pm CpG did not express CCR9 and CpG alone

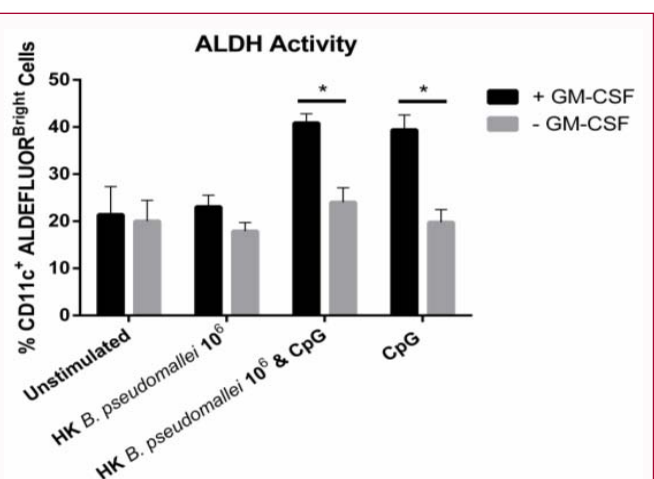


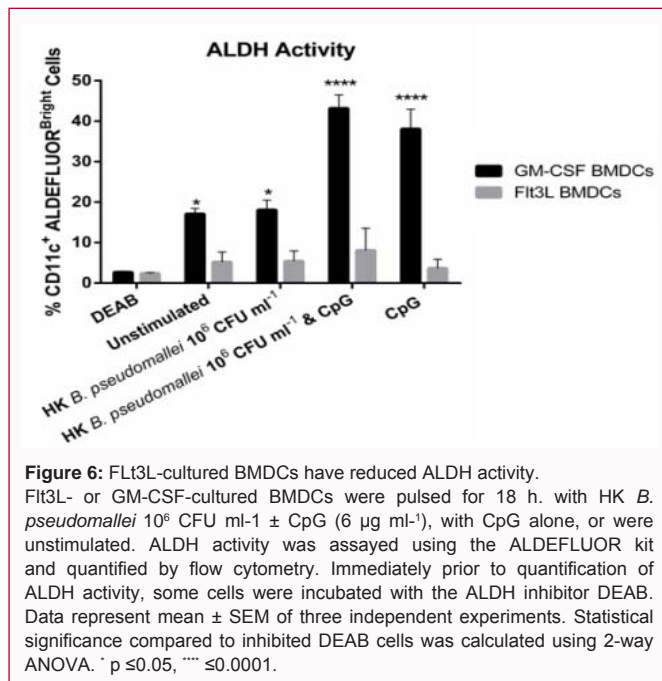
Figure 5: BMDCs sub-cultured without GM-CSF have reduced ALDH and the ALDH-inducing activity of CpG is lost. BMDCs cultured in GM-CSF were harvested from culture and washed twice in PBS before being sub-cultured for 24 h with or without GM-CSF (20ng ml^{-1}). BMDCs were pulsed for 18 h. with HK *B. pseudomallei* 10^6 CFU ml^{-1} \pm CpG (6 $\mu\text{g ml}^{-1}$), with CpG alone, or unstimulated. ALDH activity was quantified using the ALDEFLUOR kit. ALDH activity within the cells was quantified by flow cytometry. Data represent mean \pm SEM of three independent experiments. Statistical significance between GM-CSF conditions was calculated using 2way ANOVA. * $p \leq 0.05$.

had no effect (Figure 4). These data suggest that culture of BMDC in GM-CSF and not FLT3L contributes to the imprinting of CCR9 expression on co-cultured Tcells and that the addition of CpG to the activating medium with heat-killed *B. pseudomallei* significantly enhances this effect.

In vivo, the expression of CCR9 on T-cells would facilitate their migration to the gut where RA is a signalling factor. To investigate the observed synergism between CpG and GM-CSF augmentation of CCR9 expression, the metabolism of BMDC cultured initially in GM-CSF and then sub-cultured with or without GM-CSF for 24h prior to activation with heat-killed *B. pseudomallei* \pm CpG, was determined by assay of ALDH activity, an enzyme in the RA secretion pathway [31]. ALDH activity in BMDC pulsed with heat-killed *B. pseudomallei* alone, did not differ from that of unstimulated BMDC, whether or not the BMDC were cultured and sub-cultured in GM-CSF (+GM-CSF) or only cultured initially in GM-CSF (-GM-CSF) (Figure5). However when CpG was added to heat killed *B. pseudomallei* in the medium, BMDC +GM-CSF had significantly enhanced ALDH activity compared with BMDC - GM-CSF ($p < 0.05$). CpG alone, in the absence of heat killed *B. pseudomallei*, significantly enhanced ALDH activity in BMDC+ GM-CSF, compared with BMDC -GM-CSF ($p < 0.05$). By comparison, Flt3L-cultured BMDC had significantly reduced ALDH activity, even when activated with CpG ($p < 0.0001$) (Figure 6). Thus GM-CSF is the critical factor required to enhance ALDH in BMDC and CpG is synergistic with GM-CSF in inducing ALDH activity in the RA production pathway and the imprinting of gut-homing CCR9 expression on *B. pseudomallei*-activated T-cells.

The effect of pre-conditioning BMDC with tissue factors on their ability to imprint CCR9 on T-cells

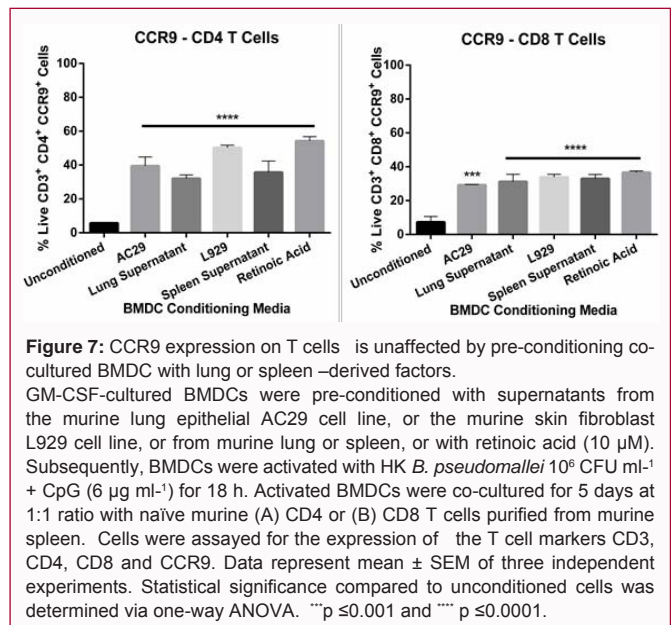
To determine whether the pre- conditioning of GM-CSF-cultured BMDCs with tissue factors other than gut, could reduce their ability to imprint gut-homing CCR9 expression on T cells, the BMDC culture media were supplemented with supernatants from murine cell lines and tissues. Thus supernatants from the murine lung cell line AC29, from the murine skin fibroblast cell line L929, from



murine whole lung, murine whole spleen, or RA, were added to the BMDC culture media for 24 h, prior to pulsing the BMDC with heat killed *B. pseudomallei* and CpG. BMDCs conditioned and activated in these ways were washed and then co-cultured with either naïve CD4+ or naïve CD8+ murine T cells for a period of 5 days. Following co-culture, cells were assessed for viability and T cells were identified based on expression of CD3 and CD4 or CD8. Finally, expression of three chemokine receptors, CCR4, CCR9 and CCR10, indicative of lung, gut and skin homing potential respectively, was assessed on T cells and compared with control co-cultures in which BMDCs had not been exposed to any tissue microenvironment prior to activation (unconditioned) (Figure 7). The migration markers CCR4 and CCR10 were not detected on any T-cells exposed to the activated BMDC (data not shown). Compared with unconditioned co-cultures, CCR9 expression on CD4+ T-cells was significantly enhanced by co-culture with BMDC conditioned with RA (as positive control) (p<0.0001). Surprisingly, however, the co-culture of CD4+ T-cells with BMDC conditioned with supernatants from the AC29 or L929 cell lines, or either spleen or lung tissues, did not diminish CCR9 expression, but instead resulted in a significant up regulation of CCR9 expression (p<0.0001). Although the percentage of CD8+T-cells expressing CCR9 was lower than that of CD4+ T-cells, CCR9 expression in these cells was also significantly up regulated by co-culture with BMDC conditioned by supernatants from lung cells (p<0.001), spleen and RA (p<0.0001), compared with T-cells exposed to unconditioned BMDC.

Discussion

The ability of DCs to elicit an immune response has resulted in the use of DCs as a therapeutic cancer vaccine and has highlighted their application in the prophylaxis of infectious disease [32-35]. We also have used *ex vivo* matured and activated BMDC to immunize naïve animals against bacterial infections by adoptive transfer [25,26]. In this context it is desirable to replicate the conditioning and activation responses naturally induced by DCs *in vivo*. Here we have used a BMDC population which is CD11c+ CD11b+ CD103- B220- which, if applied to DCs derived *in vivo* is a phenotype consistent with cDC2



cells [11,13]. Functionally, cDC2 cells are believed to be more efficient in the activation of CD4+ T cells compared to other DC subsets [13]. However, *ex vivo*-matured BMDCs expressing a phenotype consistent with cDC2 cells may not develop the same functional properties as cDC2 cells arising naturally *in vivo* and so the functionality of these BMDCs necessitated further investigation.

BMDCs cultured in GM-CSF were activated by pulsing with heat killed *B. pseudomallei* or with the membrane protein from *B. pseudomallei*, LolC, together with CpG, a potent TLR9 agonist [36]. In the absence of CpG, heat killed *B. pseudomallei* at a concentration of 10⁶ CFU ml⁻¹ enhanced expressions of co-stimulatory molecules and MHC-II but failed to induce significant cytokine production, whilst LolC was poorly activating. LolC, although identified to contain T-cell epitopes [37,38], does not appear to be an immunodominant antigen [39]. There were no qualitative differences in the activation of GM-CSF-cultured BMDCs stimulated with CpG alone or in combination with heat killed *B. pseudomallei*, but the combined effect was to enhance the expression of co-stimulatory molecules in addition to increasing secretion of IL-6 and TNF after 6 h. The expression of CCR7 on GM-CSF cultured BMDC, was enhanced by heat-killed *B. pseudomallei* + CpG, or by CpG alone, indicating a significant increase in the lymph node migratory potential of these activated BMDC and suggesting that CpG can directly induce CCR7 expression when GM-CSF is present.

The combined influence of GM-CSF and CpG on BMDC is potent and may explain our previous observation that mice receiving GM-CSF-cultured BMDCs, activated with heat killed *B. pseudomallei* and CpG, by adoptive transfer prior to pathogen challenge had 90% survival, whereas the omission of CpG from the BMDC -activating medium reduced protective efficacy to 70% [25].

Having determined the essential requirement for CpG in the successful activation of DC *ex vivo*, we next determined whether the ways in which DC were cultured affected their ability to activate and imprint migratory properties on naïve co-cultured T-cells. Murine BMDC were cultured in either GM-CSF or Flt3L prior to co-culture with T cells. Whilst GM-CSF-cultured BMDCs had conserved ALDH activity, which is required for RA secretion [40,41], Flt3L-cultured

BMDCs had significantly reduced ALDH activity. ALDH activity in BMDC cultured in either condition was unaffected by pulsing with heat killed *B. pseudomallei*. The additional incorporation of CpG into the medium had the effect of significantly enhancing ALDH activity in GM-CSF -cultured BMDC, but had no such effect upon Flt3L-cultured BMDCs. For GM-CSF-cultured BMDCs, CpG alone was as effective as the combination in significantly increasing ALDH activity, whilst CpG alone did not enhance ALDH activity in FLT3L-cultured BMDCs. Whilst the functional advantage in gut-derived CD103+ DC being able to express ALDH activity and produce RA constitutively is clear, GM-CSF has previously been reported to induce ALDH activity in CD103- BMDC and spleen-derived DC [40]. Furthermore, it has been shown that GM-CSF could trigger FLT3L-cultured BMDC to express ALDH activity and that this was enhanced by IL-4 stimulation as well as by TLR ligands such as CpG and Poly I: C [40]. From the current data, it is clear that culturing BMDC in GM-CSF is essential to maximize ALDH activity and that the inclusion of CpG in the media has a synergistic effect on this metabolic activity of DC in the RA secretion pathway. When BMDCs which had been cultured in GM-CSF or FLT3L and then activated by stimulating with heat killed *B. pseudomallei* and CpG were co-cultured with naïve T-cells, CCR9 expression was observed on T-cells exposed to GM-CSF-cultured BMDC, but not on T-cells exposed to FLT3L-cultured BMDC. This suggests that the combination of GM-CSF and CpG together imprinted a gut-homing phenotype on these T-cells.

It was of interest to determine whether the exposure of GM-CSF -cultured BMDC to factors derived from different tissues, prior to activation with heat killed *B. pseudomallei* and CpG, could induce migration markers other than CCR9 on co-cultured naïve T-cells. To this end, GM-CSF-cultured BMDC were exposed to lung, spleen or skin factors prior to activation. Paradoxically however, T cells co-cultured with GM-CSF-cultured BMDC, even when these had been conditioned with lung or spleen -derived media, prior to activation with heat killed *B. pseudomallei* and CpG, still upregulated CCR9. The data gained in this study show that GM-CSF and CpG have a critical role in activating BMDC optimally to induce CCR9 expression on co-cultured T-cells. GM-CSF and CpG had a synergistic effect on BMDC, to up regulate ALDH activity in the RA secretion pathway, thus imprinting gut-homing on co-cultured T-cells. Other gut-resident F4/80+ macrophages have been shown to secrete GM-CSF *in vivo*, which enhances ALDH activity in these cells [40].

The ability of lung-conditioned DC to induce $\alpha\beta 7$ and CCR9 on co-cultured T cells *in vitro* and *in vivo* has been reported by others [22] and shown to be mediated by RA and TGF β . These authors also reported that targeting lung DCs *in vivo* by an active intra-nasal immunization, induced protective immunity against the enteric pathogen, salmonella. The ability of lung DC to up-regulate gut-homing markers on effector T cells which would result in protective immunity in the gut, suggests that DCs may be involved in cross-protection of the mucosa. Thus the induction of CCR9 expression on T cells required to protect against an inhaled pathogen may indeed be advantageous. The induction of lung-homing markers such as CCR4 on T cells by the appropriate conditioning of DC has also been reported [20]. Lung DC-activated T-cells which were adoptively transferred trafficked to the lung more efficiently than T-cells which had been activated with spleen or mesenteric lymph node-derived DC and conferred greater protection against a subsequent intranasal challenge with influenza virus [20]. The generation of T cells devoid of CCR9 by exposure to FLT3L-cultured BMDC, suggests that it

would be feasible to induce CCR4 expression on T-cells *in vitro* by the alternative conditioning of BMDC in lung- derived media prior to T cell activation. The impact in a respiratory infection model of using DC conditioned to imprint lung -homing on effector T-cells, will be of interest.

Materials and Methods

Animals

Female BALB/c mice (6-10 weeks of age) were purchased from Charles River and held in specific pathogen free facilities in groups of 10 with free access to food and water and environmental enrichment. Mice were acclimatized to the facility for at least 5 working days before use. Schedule 1 culls were performed by cervical dislocation in accordance with the Animal (Scientific Procedures) Act 1986 by fully trained and competent members of staff. The procurement of mice to provide tissue samples for this study was approved by the Dstl IACUC the for UK Home Office project license. No procedures were performed on live animals.

Burkholderia pseudomallei

Viable *B. pseudomallei* K96243 were harvested and washed three times by centrifugation before being resuspended in PBS. The bacterial suspension was inactivated by incubation in a water bath for 4 hours at 80° C with occasional shaking. Inactivation was confirmed by inoculating 10 ml broths with heat-killed suspension and incubating at 37° C for one week. L-agar plates were subsequently inoculated with the entire broth and incubated for a further 7 days. An absence of growth indicated the bacterial suspension to be inactivated.

Bone Marrow- cultured dendritic cell preparations

Murine BMDC were prepared using modifications of published methods [29]. Murine bone marrow was flushed by syringe from rear fibulae and tibiae. Cells were then passed through a 40 μ m cell strainer to achieve a single cell suspension. The suspension was then incubated with red blood cell lysis buffer (Sigma) for 90 seconds before cells were washed in PBS. Cells were seeded into 6-well plates at 1×10^5 cells ml^{-1} in 6ml of complete media comprised of RPMI-1640 (Thermo Fisher Scientific) supplemented with 10% heat inactivated fetal bovine serum (FBS, Thermo Fisher Scientific), 1% penicillin-streptomycin-glutamine (Pen/Strep/Glu, Thermo Fisher Scientific), 50 μ M 2-mercaptoethanol (2-ME, Thermo Fisher Scientific). Subsequently 3ml of media were removed on days 3 and 5 and replaced with 3ml of fresh complete media supplemented with 20ngml⁻¹ granulocyte-macrophage stimulating factor (GM-CSF) (R&D Systems). BMDC were harvested on days 6/7. Where BMDC had been cultured in GM-CSF, they remained in GM-CSF- supplemented complete media for all subsequent assays, unless otherwise indicated.

Previously published methods were adapted to generate Flt3L-cultured BMDC [30]. Flt3L-secreting melanoma cells were seeded in 125 cm^2 culture flasks at a cell density of 3000 cells/ cm^2 in complete media supplemented as above with FBS, Pen/Strep/Glu and 2-ME. Cells were cultured until confluent, when the culture supernatant was removed and centrifuged at 200g for 5 minutes. The resulting supernatant was then filter -sterilised through a 0.2 μ m filter and used at a dilution of 15% (v/v) in complete media.

Murine bone marrow was flushed via syringe from rear fibulae and tibiae to release cells which were passed through a 40 μ m cell strainer to achieve a single cell suspension. The suspension was then incubated with red blood cell lysis buffer (Sigma) for 90 seconds

before cells were washed in PBS. Cells were seeded into 6-well plates at 1×10^6 cells ml^{-1} in 5ml of complete media containing Flt3L and cultured for 9 days.

After 6-9 days of culture, BMDCs were harvested by gentle washing. Cells were re-suspended in complete media as above, and with either GM-CSF or Flt3L, at 1×10^6 cells ml^{-1} and pulsed with heat-killed *B. pseudomallei* K96243 at various concentrations, or with recombinant LolC (Lionex GmbH) from *B. pseudomallei* at $10 \mu\text{g}$ ml^{-1} for 2, 6, 18 or 24 h. CpG 1826 (*Invivogen*) was added to indicated pulse cultures at a concentration of $6 \mu\text{g}$ ml^{-1} .

BMDC cultured in either condition were used whole in all assays, however all the analysis was based on detection of CD11c expression, with or without co-stimulatory markers, and of % CD11c+ cells responding, by flow cytometry (BD FACS-CANTO II). At 9 days of culture approximately 45% of FLT3L-cultured BMDC were CD11c+, compared with 65% of GM-CSF-cultured BMDC being CD11c+ at 6-7 days.

Cytokine analyses

Mouse Inflammation Cytokine Bead Array (BD Biosciences) assay was performed as per manufacturer's instructions. Briefly, cell culture supernatants were incubated with cytokine capture beads in addition to phycoerythrin (PE) conjugated detection reagent, specific for each cytokine, for 2 h. Beads were then washed to remove excess detection reagent and fluorescence intensity quantified by flow cytometry, according to the manufacturer's instructions.

Preparation of tissue factor conditioning media

The murine lung epithelial cell line AC29 (Public Health England Culture Collections) and the murine subcutaneous cell line L929 (Public Health England Culture Collections) were individually seeded in 125 cm^2 culture flasks at a cell density of 2500 cells/cm^2 in complete media, as described above. Cells were cultured until confluence was achieved, when the conditioned media were removed, centrifuged at $200g$ for 5 minutes, filter-sterilized through a $0.2 \mu\text{m}$ filter, aliquoted and stored at -80°C until required. Freshly isolated murine lung or spleen was dissected into equal segments and suspended in complete media in an untreated cell culture flask. Suspended segments were agitated periodically. After 30 h., the media were removed, centrifuged at $200g$ for 5 minutes, filter sterilized through a $0.2 \mu\text{m}$ filter, aliquoted and stored at -80°C until required. Conditioned media (CM) were diluted 1:1 with fresh complete media immediately prior to use.

On day 5 of BMDC culture, 3ml of media were removed and replaced with 3ml of GM-CSF supplemented CM. Once introduced to CM, BMDC remained in CM for all subsequent assays.

T-cell isolation

Naïve CD4 and CD8 T cells were isolated from murine spleen using Miltenyi MACS Naïve CD4 and Naïve CD8 T cell isolation kits (Miltenyi Biotec Ltd, Surrey UK). Purification was conducted according to the manufacturer's instructions. Briefly, a single cell suspension was achieved by passing spleens through a $45 \mu\text{m}$ cell sieve. Cells were then incubated with red cell lysis buffer (Sigma) for 90 seconds after which remaining cells were resuspended in PBS supplemented with 0.5% heat inactivated fetal bovine serum (Thermo Fisher Scientific) and 2 mM EDTA. Cell suspensions were then incubated for 5 minutes with either biotinylated anti-CD4 or anti-CD8, and then for a further 10 minutes with anti-biotin Micro Beads and CD44 Micro beads. Following labelling, cells were washed

to remove unlabelled antibody and run on LS MACS columns. Eluent was collected, representing unlabelled pure naïve CD4 or CD8 T cell suspensions. T cell purity was validated for all preparations.

BMDCs and T cell populations were established as described above. BMDCs used in co-cultures were harvested on day 6 following isolation from bone marrow and pulsed for 18 hours with heat-killed *B. pseudomallei* K96243 at 10^6 colony forming units (CFU) ml^{-1} with or without CpG 1826 (*Invivogen*) at $6 \mu\text{g}$ ml^{-1} as indicated. T cells and BMDCs were seeded in a 96-well plate at a 1:1 ratio (5×10^4 total cells per well) in $200 \mu\text{l}$ of complete media and cultured for 5 days.

Assay for aldehyde dehydrogenase (ALDH) activity

Analysis of cellular ALDH activity was estimated using the ALDEFLOUR assay kit (Stem Cell Technologies, Canada), as per the manufacturer's instructions. Briefly, cells were suspended in ALDEFLOUR buffer at a 10^6 cells ml^{-1} . ALDH inhibitor DEAB ($25 \mu\text{M}$) was added to some cells. Cells were then incubated with activated ALDEFLOUR substrate (BAAA; $1.5 \mu\text{M}$) for 30 minutes at 37°C . Cells were then washed in assay buffer prior to surface immune fluorescence staining. Subsequent staining and flow cytometric analysis was carried out in ALDEFLOUR assay buffer.

Flow cytometry

Prior to staining, cells were washed and re-suspended in PBS and incubated for 30 minutes with the indicated fluorophore-conjugated antibody. Cells were then washed in PBS and fixed in 1% paraformaldehyde. Following staining, sample data were acquired on a BD FACScan to II using BD FACS Diva acquisition software. Data analysis was carried out using Flow Jo software (Flow Jo, Oregon USA).

The following antibodies were used and purchased from Biolegend Inc: FITC anti-CD11c (clone N418), APC anti-CD40 (clone 3/23), PE anti-CD80 (clone 16-10A1), PE/Cy7 anti-CD86 (clone GL-1), Per CP/Cy5.5 anti-MHC-II (clone M5/114.15.2), PE anti-CD11c (clone N418), PE/Cy7 anti-CD11b (clone M/170), FITC anti-B220 (clone RA3-6B2), FITC anti-CD103 (clone 2E7), Pacific Blue anti-CD11c (clone N418), PE anti-CCR7 (clone 4B12), Brilliant Violet 421 anti-CD3 (clone 145-2C11), PE anti-CD4 (clone GK1.5), APC anti-CD8a (clone 53-6.7) Per CP/Cy5.5 anti-CD4 (clone RM4-4), Per CP/Cy5.5 anti-CD8 (clone YT5156.7), PE anti-CCR4 (clone 2G12) and PE/Cy7 anti-CCR9 (clone CW-1.2). APC anti-CCR10 (clone #248918) was purchased from R&D Systems. Viability assays were conducted using Invitrogen LIVE/DEAD Fixable Yellow Dead Cell Stain Kit.

Statistical analysis

All statistical analysis was performed using Graphpad Prism v.6 software, with p values < 0.05 considered to be significant.

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