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Selective elevation of circulating CCL2/MCP1 levels in patients with longstanding post-vaccinal macrophagic myofasciitis and ASIA.

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ABSTRACT

Several medical conditions sharing similar signs and symptoms may be related to immune adjuvants. These conditions described as ASIA (Autoimmune/inflammatory Syndrome Induced by Adjuvants), include a condition characterized by macrophagic myofasciitis (MMF) assessing long-term persistence of vaccine derived-alum adjuvants into macrophages at sites of previous immunization. Despite increasing data describing clinical manifestations of ASIA have been reported, biological markers are particularly lacking for their characterization and follow up. We report an extensive cytokine screening performed in serum from 44 MMF patients compared both to sex and age-matched healthy controls and to patients with various types of inflammatory neuromuscular diseases. Thirty cytokines were quantified using combination of LuminexR technology and ELISA. There was significant mean increase of serum levels of the monocyte-chemoattractant protein 1 (CCL2/MCP-1) in MMF patients compared to healthy subjects. MMF patients showed no elevation of other cytokines. This contrasted with inflammatory patients in whom CCL2/MCP-1 serum levels were unchanged, whereas several other inflammatory cytokines were elevated (IL1 β , IL5 and CCL3/MIP1 α). These results suggest that CCL2 may represent a biological marker relevant to the pathophysiology of MMF rather than a non specific inflammatory marker and that it should be checked in the other syndromes constitutive of ASIA.

KEYWORDS

macrophagic myofasciitis

inflammatory cytokine

cytokinome

CCL-2/MCP1

vaccine adjuvant

alum

INTRODUCTION

During the last century, billions of people have received a variety of vaccines leading to virtual eradication of several infectious diseases. The extending scope of possible immunization strategies has promoted vaccine as a privileged weapon against a variety of diseases. As a general rule, vaccine safety has been considered excellent in the overall population [1] but adverse reactions to vaccines were long suspected to occur in a very small minority of cases [2]. Their mechanism usually remains unclear. Vaccine adverse reactions could represent autoimmune phenomena similar to those following natural infections [3]. For example, autoimmune diseases may result from: (a) molecular mimicry between an infectious agent and host antigenic components; (b) epitope spreading, with progressive appearance of immune responses to different epitopes on the same or on another antigen; (c) release of cryptic epitopes or previously hidden auto-antigens; (d) bystander activation of T cells and polyclonal activation of B-cells; (e) superantigenic T cell activation by infectious products cross-linking the T-cell receptor and MHC molecule independently of specific antigen recognition; and (f) anti-apoptotic effects on autoreactive cells. Besides classical explanations centered on vaccine antigens [4], attention has progressively focussed on possible implication of some adjuvants used to boost the immune response, including aluminium oxyhydroxide or alum [5,6] and squalene [7,8]. It is likely that a fine balance exists between the efficacy of vaccine adjuvants and their potential toxicity, and these may be one and the same effect [9]. Adjuvants may exert their immunostimulatory effect by many different actions, including: (a) protection of antigens resulting in prolonged delivery; (b) induction of prompt vaccine particle phagocytosis by dendritic cells and macrophages with up-regulation of their antigen-presenting function; (c) translocation of antigens to lymphoid organs where the primary activation of naïve T cells takes place; (d) amplification of the inflammatory reaction in the injection site and its draining lymph nodes through interaction with pattern recognition receptors and

release of inflammatory cytokines; and (e) priming of B cells in spleen [9,10,11]. Vaccine adverse effects include specific autoimmune diseases that are easily identified using well established criteria but also ill-defined conditions usually manifesting by symptoms such as myalgia, arthralgia, chronic fatigue and the appearance of autoantibodies [12]. Yehuda Shoenfeld had the great merit to tentatively delineate a syndrome entitled "Autoimmune (Autoinflammatory) syndrome Induced by Adjuvants" (ASIA) in order to alert physicians when the above-mentioned symptoms do appear following vaccination [13]. As noted previously by others [14] he pointed out remarkable similarities between different syndromes linked to vaccine adjuvants, including the one associated with macrophagic myofasciitis, a lesion assessing longterm persistence of vaccine-derived alum into macrophages at site of previous immunization [15] and the so-called Gulf war syndrome that developed in soldiers exposed to intense immunization protocols [16] with vaccines containing alum [17] and possibly squalene [7]. Yehuda Shoenfeld also noted similarities with siliconosis, a disease complex observed in patients with leaky breast silicone implants which was long attributed to deleterious adjuvanticity of silicone particles [18,19]. He unified, under the heading of ASIA, the 3 syndromes with other post-vaccinal events, and proposed a set of major and minor diagnostic criteria as a guide for characterization and follow-up of these conditions [13]. These criteria still need validation but, notably, ASIA grab the attention of the international medical community rapidly after its delineation [20,21,22] pointing to a need in the field [15, 23]. In addition to clinical manifestations, it could prove very useful to identify biomarkers that may either help unify the ASIA concept in the future or, alternatively, point out subtle differences among its constitutive syndromes. In the present study we performed extensive cytokine screening in serum of patients with alum-related MMF. This allowed us to detect selective increase of the monocyte chemotactic protein 1/ C-C motif ligand 2 (MCP-1/CCL2), pointing out this chemokine as a first candidate biomarker to be tested in other ASIA subsets.

PATIENTS AND METHODS

1- The MMF Cohort. Analysis of our files yielded 583 patients with stereotypical MMF detected by muscle biopsy from 1994 to 2012. Patients were either investigated and biopsied in our Reference Centre at Créteil (n=352), or referred for follow-up or investigation after MMF detection by the other myopathologic centers located in Bordeaux, Marseille, and Paris (n= 231) that described the entity with us [24]. For the present study, prevalence of core clinical symptoms were determined in 105 unselected consecutive patients, 25 males and 80 females (age range 12-64 years), who benefited from systematic evaluation of their brain function in the Cognition Center of Créteil, using a comprehensive battery of neuropsychological tests detailed elsewhere [25,26]. The persistent MMF lesion consisted in stereotyped accumulations of large particle-loaded macrophages without multinucleated giant cell formation, associated with T and B cells. Granular inclusions were hematein-positive in cryostat sections, grey after formalin fixation, PAS positive, and green-fluorescent on Morin stain for aluminium (Figure 1). MMF was always detected in the deltoid muscle with a median delay of 6.5 years elapsed from the last alum-containing vaccine administration and the biopsy. Patients had received a combination of various vaccines within 10 years before biopsy: anti-hepatitis B virus vaccine administered in 3 shots was implicated in a vast majority of patients (89.7% of patients), followed by tetanus toxoid (67.9%), and anti-hepatitis A virus (14.1%) vaccines. Main symptoms declared by patients were arthromyalgias (96/105: 91.4%), fatigue (88/105: 83.8%), and cognitive alterations affecting attention and memory (102/105: 97.1%). Neuropsychological evaluation confirmed abnormal cognitive status in 93/105 (88.6%) of patients, with a pattern suggestive of subcortico-frontal alterations in 89/105 (85%). Onset of these manifestations was typically delayed from immunization. Thus 3 Major Criteria proposed for ASIA [13] were met, including (1) “exposure to adjuvant prior to clinical

manifestations” (i.e. aluminium oxyhydroxide), with a previously reported median time of 7 months (range 0.5–84) elapsed from last vaccine administration to initial systemic symptoms, and 11 months (range 0–72) for first myalgia [15]; (2) “the appearance of typical clinical manifestations” including: myalgia; arthralgias; chronic disabling fatigue; unrefreshing sleep or sleep disturbances; cognitive impairment and memory loss [27]; and, less often, overt neurological manifestations associated with demyelination [28] ; and (3) “typical biopsy of involved organs” , i.e. MMF [24] . Notably, in contrast to the leaky silicone breast implant-associated ASIA, the criterion “removal of inciting agent induces improvement” could not be met in our patients subjected to multiple vaccine shots. A variable proportion of MMF patients also met minor criteria for ASIA, including (1) “the appearance of autoantibodies”, (2) “other clinical manifestation” such as dyspnea, (3) “specific HLA”, i.e. HLADRB1; and (4) “evolution of an autoimmune disease”, i.e. mainly multiple sclerosis thyroiditis, and autoimmune myopathies [15].

2- The cytokine study subjects and controls. Chemokine serum levels were determined in 44 adult MMF patients (M/F sex ratio 16/28, mean age 46.2 ± 1.6 yrs) and in 44 normal age and sex-matched controls (M/F sex ratio 16/28, mean age 48.5 ± 0.7 yrs, $p=NS$). According to the principles expressed in the Helsinki Declaration, all patients gave written individual informed consent to participate to the study and approval was obtained from French institutional review boards (registration number 2010-A01338-31 at AFSSAPS, and 11-042 at CPP-Ile-de-France IX). MMF had received 1 to 17 i.m. alum-containing vaccine administrations (mean 5.2) within the past 10 years. Inclusion criteria were as follows: (1) onset of clinical manifestations posterior to alum-containing vaccine administration; (2) ASIA symptoms, including diffuse arthro-myalgias lasting >6 months and/or profound fatigue lasting >6 months and/or disabling cognitive deficiency affecting attention and memory; (3) histologic MMF in deltoid muscle detected by biopsy at least 18 months after the last vaccine; (4) no other disease that could explain the manifestations. Similarly to MMF patients,

healthy controls included both smokers (n=16, 1 paquet per day) and non-smokers (n=28). Ten unmatched patients with various types of inflammatory myopathies and neuropathies served as inflammatory controls.

3- Cytokine screening. Quantitation of 30 cytokines was performed on serum kept frozen at -80°C until analysis by the immunomonitoring platform of the Vaccine Research Institute of Créteil. Analysis used in combination the LuminexR technology and conventional ELISA. The Bio-Plex Pro Human Cytokine 27-plex Assay from Bio-Rad (M50-0KCAF0Y) was used following the procedure recommended by the manufacturer. This assay simultaneously detects IL-1 β , IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, basic FGF, G-CSF, GM-CSF, IFN- γ , IP-10 (CXCL10), MCP-1 (CCL2), MIP-1 α (CCL3), MIP-1 β (CCL4), RANTES (CCL5), eotaxin (CCL11), PDGF-BB, TNF- α , and VEGF. The 27plex was enriched with two simplex sets for MCP-3 (CCL7) and KC/Gro- α (CXCL1), and an ELISA kit for the detection of human Fractalkine (CX3CL1) purchased from R&D (quantikine DCX-310). Controlled intraindividual variations were <5%. Statistical analyses used Mann-Whitney U-test. $p < 0.05$ was considered significant.

RESULTS

Multiplex cytokine serum analysis showed significantly increased mean CCL2/MCP-1 levels in MMF patients with ASIA compared with healthy subjects (median = 57.7 vs. 37.9 pg/mL; mean = 64.5 \pm 32.9 vs. 38.2 \pm 11.5 pg/ml, $p < 0.0001$) (Fig. 2). Increase was unique to CCL2/MCP-1, as the other tested inflammatory cytokines, chemokines, and growth factors were either similar (IFN γ , TNF α , IL5, IL6, IL7, IL13, G-CSF, PDGF-bb, VEGF, CCL3, CCL4, CCL5, CCL7, CCL11, CXCL8, CXCL1, CXCL10, and CX3CL1, $p = \text{NS}$), or even decreased (IL1 β , IL1ra, IL4, IL10, IL12p70, IL17, FGFb, all $p < 0.05$) in MMF patients compared to healthy controls. The CCL2/MCP1 variation could not be attributed to smoking habits ($p = \text{NS}$ for all tested molecules). Inflammatory

controls showed no difference for serum CCL2/MCP-1 levels (median 47.9; mean 52.8+/- 25.9pg/mL) compared with both MMF and healthy control patients ($p=NS$). In contrast, inflammatory controls had significantly increased mean levels of several inflammatory cytokines compared to MMF patients (IL1b, IL5 and CCL3/MIP1a, all $p < 0.04$), and to healthy controls (CCL3/MIP1a, $p < 0.02$). Similarly to MMF patients, inflammatory controls had decreased mean IL17 and FGFb levels compared to healthy controls (Fig. 3).

DISCUSSION

The salient finding of this cross-sectional screening of 30 cytokines was the selective increase of CCL2/MCP-1 serum levels in MMF patients with ASIA compared to age- and sex-matched healthy individuals. Notably, similar increase of circulating CCL2/MCP1 levels was not detected in inflammatory controls, whereas other inflammatory molecules, including IL1 β , IL5 and CCL3/MIP1a levels, were higher in this group. Furthermore, MMF patients had lower mean levels of IL1b and IL1ra compared to healthy controls, pointing to CCL2/MCP-1 as a marker relevant to the pathophysiology of MMF rather than a non-specific inflammatory marker. They also had lower mean levels of IL 4 and IL10 of unknown significance.

MMF assesses long-term persistence of alum crystal agglomerates within macrophages at the site of previous i.m. immunization [5]. Such a biopersistence of alum is detected in the deltoid muscle a small minority of alum-adjuvanted vaccine receivers, strongly suggesting the influence of genetic factors, as previously documented in rats in which the lesion size conspicuously varies according to the genetic background [29]. The rarity of ASIA in the general population may also reflect specific gene-environment interactions [13]. Adjuvant effects of alum remain incompletely understood [9]. In vitro studies have shown that alum acts mainly on macrophages and monocytes, skewing monocyte differentiation toward inflammatory

DCs (iDCs) [30]. Whereas classical iDCs do not secrete CCL2, iDCs differentiating in the presence of alum produce high amounts of CCL2 from day 4 onward, and this does not appear to simply reflect particle uptake [30]. Unlike LPS which induces a wide proinflammatory response, alum does not upregulate other cytokines, if one excepts CCL4, and, therefore, likely acts through a mechanism distinct from that of danger signals [30]. In addition to monocyte/macrophage lineage cells, CCL2 is produced by many cell types, including for example endothelial, fibroblasts, smooth muscle, astrocytic, and microglial cells [31]. CCL2 acts through its receptor CCR2, which expression is restricted to certain type of cells, such as monocytes, T and NK cells [31]. CCL2 is the main chemokine responsible for recruiting inflammatory monocytes to tissues, as previously documented in both injured muscle [32] and brain [33,34]. Certain individuals are genetically programmed to produce copious amounts of MCP-1, and these individuals were reported to be at higher risk of developing severe forms of diseases implicating macrophages in their pathophysiology, including atherosclerosis [35], tuberculosis [36], sarcoidosis [37], pneumoconiosis [38], lupus erythematosus [39,40], and HIV-associated dementia [41]. In light of these reports, selective increase of CCL2/MCP1 serum levels in MMF patients suggests that functional polymorphisms in the promoter of the MCP-1 gene should be screened as possible genetic susceptibility factors to develop the condition after alum administration. For example, CCL2 expression is associated with the development of polarized Th2 immune responses [42], and both CCL2/MCP1 and Th2 cells increase in blood during normal ageing [43]. Th2 cytokines may disturb the xeno/autophagy process [44] used by macrophages to handle both infectious and non-biodegradable particles [45]. Here again, genetic susceptibility factors further impacting the xeno/autophagic machinery should be searched in MMF patients in light of recent reports linking polymorphisms in certain genes with inability of cells to clear out infectious particles in Crohn disease

[46], a chronic inflammatory gastrointestinal disease in which aluminium may also play a role [47].

In a preliminary series of mouse experiments, we observed that nanomaterials injected into muscle, including latex beads and fluorescent alum surrogates, are quickly captured by monocyte lineage cells and disseminate in the body in a CCL2-dependent manner, as demonstrated by gain and loss of CCL2 function experiments [48]. Particles are transported by MO-lineage cells to the draining lymph nodes, then reach blood and spleen, and, after a long delay and at a much lower rate, gain the brain where they accumulate and persist mainly within microglial cells. Alum adjuvant injected twice into mice at human equivalent doses to mimick vaccine exposure of individuals with Gulf war syndrome results in conspicuous neurotoxic effects, as assessed by behavioural and motor testings, and by neuropathologic evidence of microglial activation and motoneuronal death [17]. CCL2 is constitutively present in the central nervous system where it acts both as chemoattractant and neuromodulator [49]. CCL2 is an important mediator of the neuroinflammatory responses in chronic neurologic disorders such as multiple sclerosis [50], Alzheimer's disease [51] and human HIV-1-associated dementia [52]. Several CCL2 neuromodulatory effects have been documented. For example CCL2 protects against toxicity of trace metals such as methylmercury at low dose, protection ceasing and marked toxicity ensuing when exposure becomes chronic or when too much CCL2 is released by glial cells [53]. Consistently, chronic CCL2 overexpression is associated with delayed encephalopathy and impaired microglial function [54]. Moreover, CCL2 is crucially involved in the development of pain [55,56,57], CCL2-induced central sensitization to pain being mediated by increasing the activity of NMDA receptors in dorsal horn neurons [58]. In keeping with these effects, circulating CCL2/MCP1 elevation was previously reported in patients with fibromyalgia, in combination with IL8 elevation [59], which was not detected in the present study, and with mild cognitive impairment [60]. Consistently,

MMF patients have chronic myalgias combined with fatigue and mild cognitive impairment of the multi-domain type [26]. We suggest that circulating MCP-1 levels should be tested as a possible biomarker in the other ASIA subsets.

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Figure legends

Figure 1 : Morin stained section from muscle biopsy with macrophagic myofasciitis showing rounded alum agglomerates within cells. (magnification x250)

Figure 2 : Serum CCL2-MCP1 levels in MMF patients compared to control non-smoking and smoking healthy subjects and inflammatory patients. CCL2-MCP1 is selectively increased in MMF patients and unchanged in inflammatory patients.

Figure 3 : Serum cytokine levels in MMF patients, control healthy subjects and inflammatory patients. MIP-1 α , 1L-1 β and IL-5 are significantly increased in inflammatory patients but similar to healthy controls in MMF patients.

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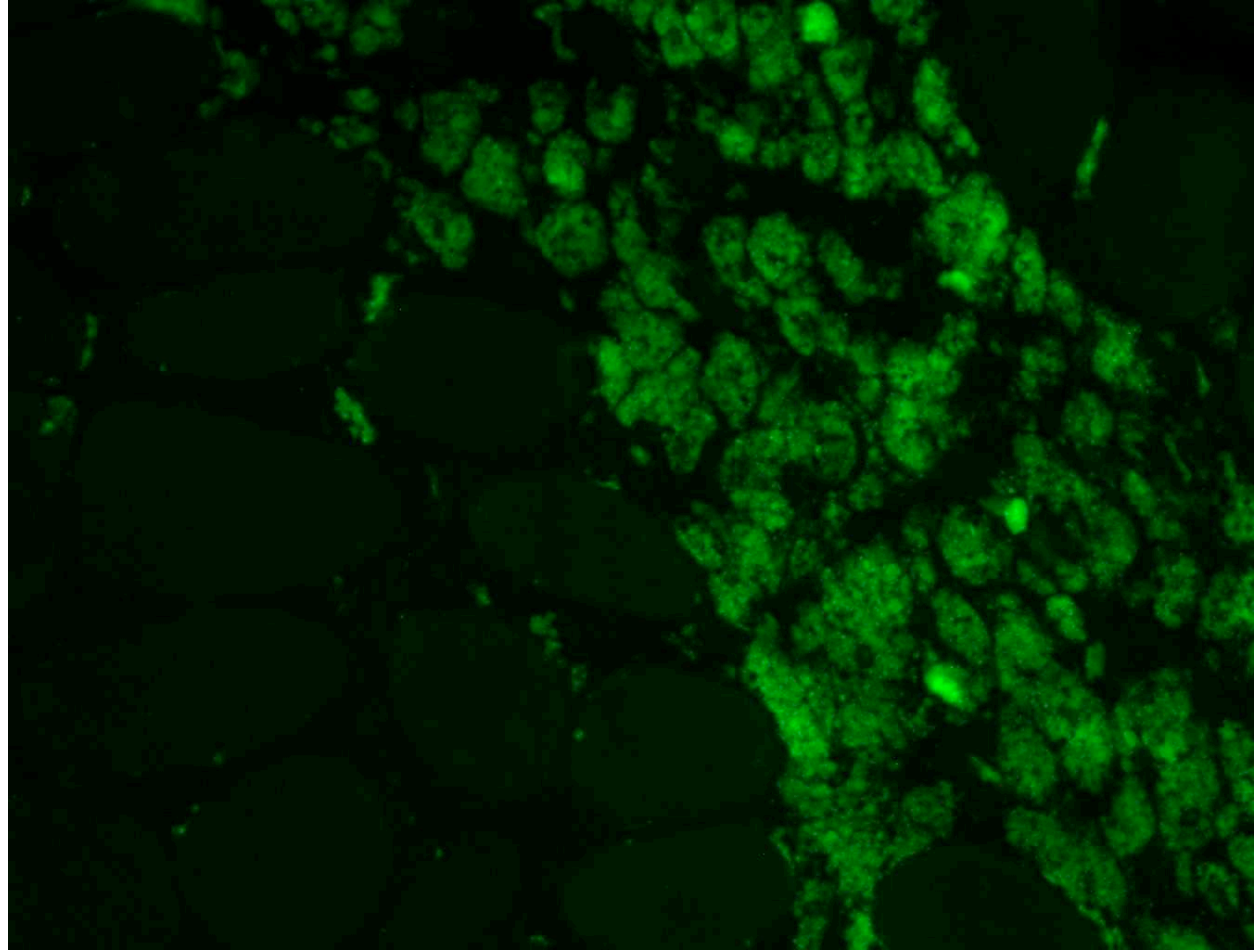


Figure 1

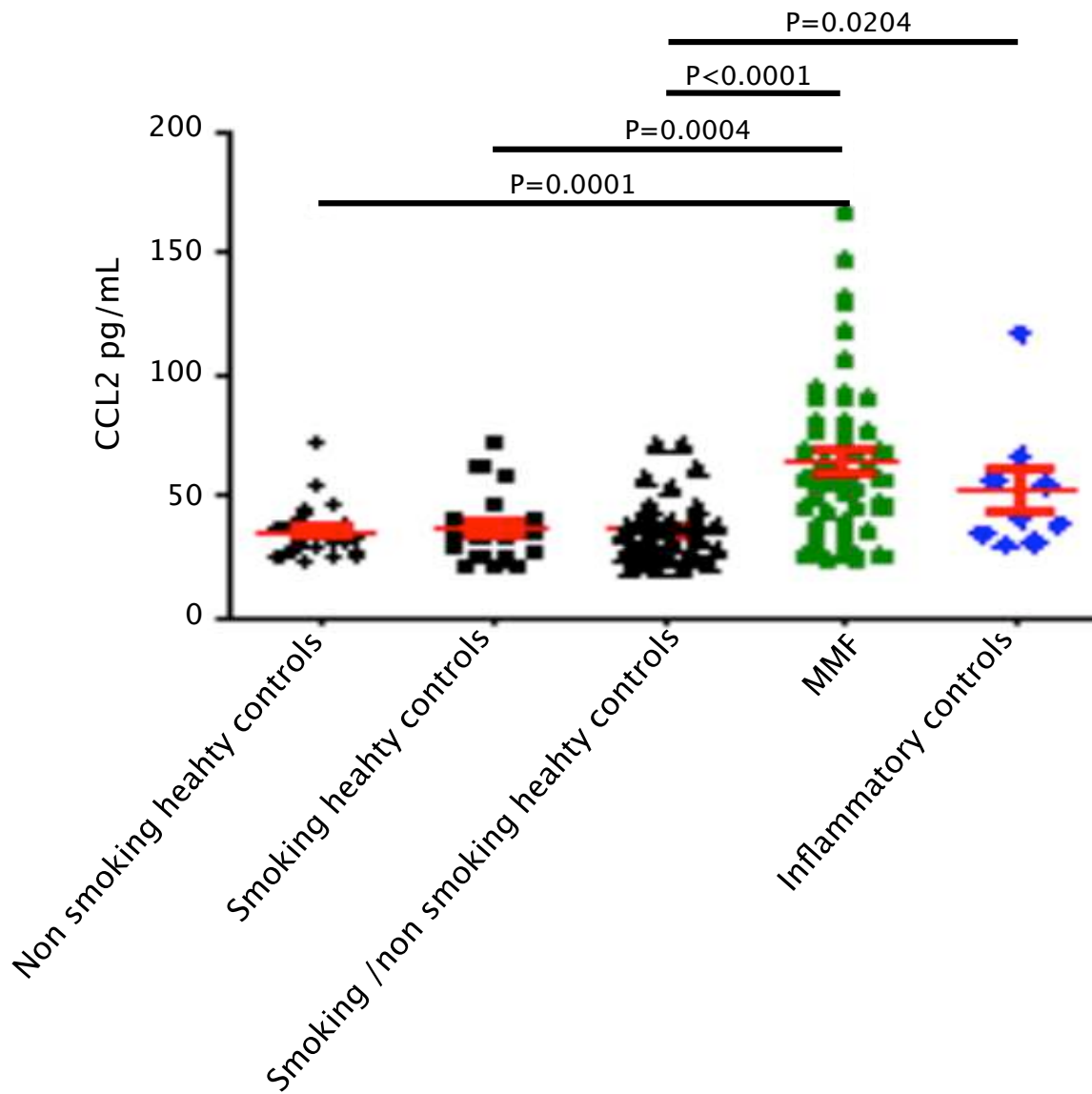


Figure 2

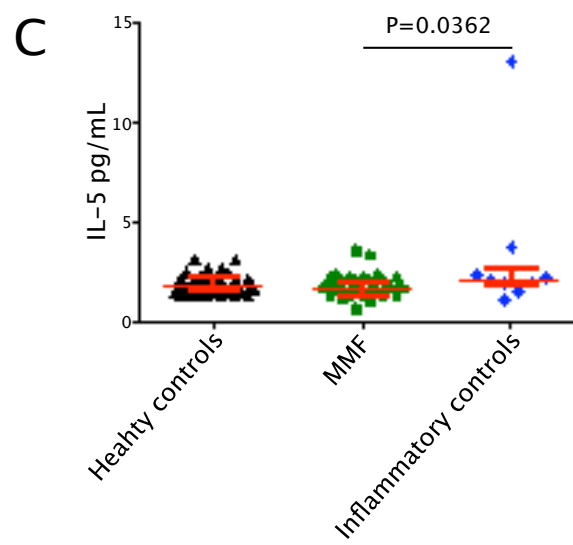
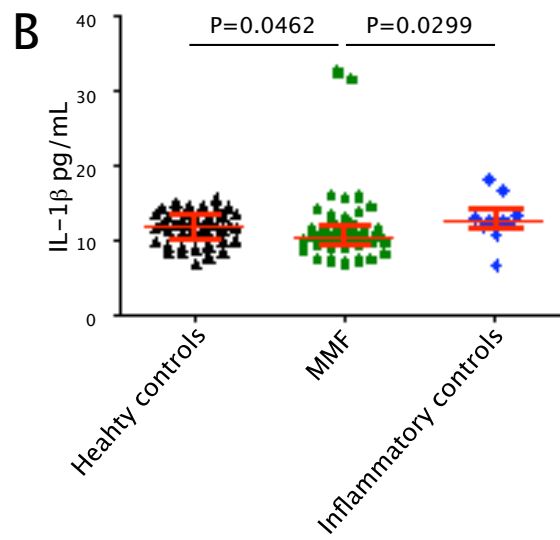
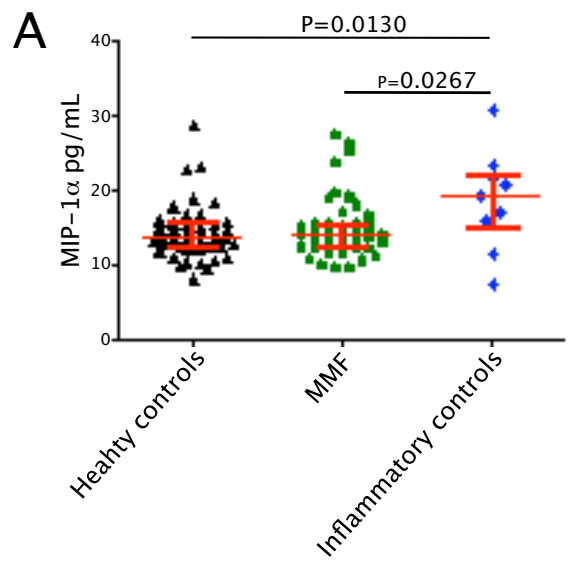


Figure 3