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Impact of IgA isoforms on their ability to activate dendritic cells and to prime T cells

Short: IgA is a mediator of adaptive immunity

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Abstract

Human IgA could be from different isotypes (IgA1/IgA2) and/or isoforms (monomeric, dimeric or secretory). Monomeric IgA mainly IgA1 are considered as an anti-inflammatory isotype whereas dimeric/secretory IgA have clearly dual pro- and anti-inflammatory effects. Here, we show that IgA isotypes and isoforms display different binding abilities to FcαRI, Dectin-1, DC-SIGN and CD71 on monocytes-derived dendritic cells (moDC). We describe that IgA regulate the expression of their own receptors and trigger modulation of moDC maturation. We also demonstrate that dimeric IgA2 and IgA1 induce different inflammatory responses leading to cytotoxic $CD8^+$ T cells activation. moDC stimulation by dimeric IgA2 was followed by a strong pro-inflammatory effect. Our study highlights differences regarding IgA isotypes and isoforms in the context of DC conditioning. Further investigations are needed on the activation of adaptive immunity by IgA in the context of microbiota/IgA complexes during Antibody-Mediated Immune Selection (AMIS).

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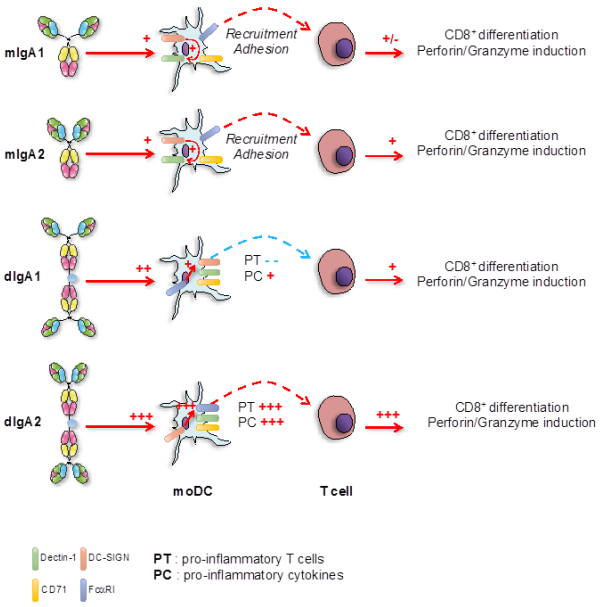
IgA isotypes and isoforms bind on different receptors on moDC and regulate their maturation. Monomeric IgA have low capacity to promote DC activation. Dimeric IgA2 promote inflammatory responses leading to cytotoxic CD8+ T cells activation.

Graphical abstract



Keywords: IgA, isotype, isoform, secretory, dendritic cells

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Introduction

With a production of around 75 mg/kg/day, human immunoglobulin A (IgA) are the most produced antibodies in the body by comparison with IgG (~22 mg/kg/day) or with IgM (~7 mg/kg/day). In particular, IgA are the main isotypes found in mucosal fluids, such as vaginal and gastric secretions, saliva and colostrum [1]. IgA can be produced as monomeric (mIgA) or dimeric (dIgA) forms after binding with J-chain. The secretory forms (SIgA) are generated by recovering the secretory component (SC) from the polymeric immunoglobulin receptor (pIgR) during epithelial transcytosis [2]. IgA are mainly monomeric in the serum (80% to 99% depending on the individual) [3] whereas mucosal IgA are predominantly dimeric. Human IgA1 and IgA2 differ by the size of their hinge region between the Cα1 and Cα2 regions, their shape, their amino acid sequence and especially their glycosylation profiles [4] [5]. IgA1 contain two N-linked glycosylation and five O-glycosylation sites whereas IgA2 are devoid of O-glycosylation but contain two additional N-glycosylation sites [5]. The proportion of IgA1 is globally higher (~85%) as compared to IgA2, especially in the blood, the spleen (~95%), the nasal mucosa (~96%), the proximal small intestinal mucosa (till 84%) or the colostrum (~65%) [5, 6]. IgA2 are preferentially found in colonic fluids (~65%) and the female reproductive system [5]. Human IgA were described to bind several receptors such as the Fcα receptor I (FcαRI) [7], the transferrin receptor (CD71) [8], Dectin-1 (Dectin-1) [9] or Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) [10]. Serum IgA are derived from plasma cells in the bone marrow whereas dIgA are produced by plasma cells from the *lamina propria* beneath the mucosa epithelium [11]. They have been extensively studied since their function, called immune exclusion, is one of the first lines of protection in mucosal surfaces. This consists of commensal or pathogen bacteria coating by respectively low- or high-affinity IgA, controlling their entrance across the intestinal epithelium. Nevertheless, in addition to this anti-inflammatory function, dIgA and SIgA were recently described as pro-inflammatory actors in mucosa by recruiting neutrophils [12, 13] or by transporting mucosal antigens (Ag) to Ag presenting cells (APC) such as macrophages and dendritic cells (DC) in the *lamina propria* or Peyer's patches. In such case, inflammation can be induced after IgA recognition by receptors such as the transferrin receptor (transferrinRCD71) [1]. Given the role of IgA in the microbiota selection during the first years of life, it is suggested that the functions of IgA are tightly regulated between defense mechanisms against pathogens and the antibody-mediated immunoselection (AMIS) [14-16]. mIgA are mainly described for their anti-inflammatory effect in the serum [17] even if a study recently described a pro-inflammatory effect mediated through FcαRI [18]. Roles of IgA are highly diverse depending on the environment and would deserve to be investigated in details. Differential interactions of IgA isotypes and isoforms with their cognate receptors, such as C-type lectin receptors, may explain the diversity of related

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immune responses. In addition, location of dIgA in the mucosa areas compared to serum monomeric IgA could result in different immune functions. Finally, both IgA1 and IgA2 are found in mucosal secretion, raising the question of their respective local functions. Here, we aimed to clarify the difference between IgA1 and IgA2 in their ability to bind to DC and to induce pro- or anti-inflammatory response. The immunostimulatory effects of polymeric IgA *vs* monomeric IgA were also compared.

Results

IgA1 and IgA2 bind to different receptors on moDC populations.

To determine whether the binding capacity of IgA isotypes and isoforms on APCs was different, the interaction of monoclonal monomeric (m), dimeric (d) and secretory (S) IgA1 and IgA2 with IgA receptors on human monocyte-derived dendritic cells (moDC) was compared. Interestingly, we observed high expression of not only FcαRI, CD71, DC-SIGN but also, for the first time, Dectin-1 on the moDC surface without treatment (**Fig. 1A**). mIgA1 bound weakly but specifically to FcαRI and CD71 and mIgA2 bound specifically to FcαRI, Dectin-1 and DC-SIGN **(Fig. 1A and B)**. mIgA2/FcαRI, mIgA2/DC-SIGN, mIgA2/Dectin-1, mIgA1/CD71 co-localization present the highest staining intensity (**Fig. 1A and B**). Dimeric IgA bind with the same restricted specificity but with less avidity. Secretory IgA weakly binds to the different tested receptors (**Fig. 1A and B**). Specificity of IgA1 for CD71 and FcαRI and IgA2 for FcαRI, Dectin-1 and DC-SIGN were also confirmed using monomeric and different dimeric IgA1 and IgA2 purified from hybridomas using HELA-transfected cells with FcαRI/CD89, CD71, Dectin-1 and DC-SIGN **(Sup. Fig. 1)**. We then wondered whether mIgA uptake was affected by the profile of IgA receptors expressed on moDC **(Fig. 1C)**. Interestingly, almost no IgA^+ moDC were observed when the cells expressed only one of the IgA receptors, suggesting the requirement for at least two different receptors to significantly increase the IgA internalization by moDC (**Fig. 1C, Supplementary Fig. 3A**). In contrast, the frequency of IgA^+ cells increased 2, 3 and 4 times for moDC expressing respectively two, three and four receptors, indicating a proportional correlation between the expression of IgA receptors on DC and their ability to internalize IgA (**Fig. 1C**). A predominant role of DC-SIGN and Dectin-1 for IgA isoforms uptake was suggested since 90% of IgA^+ cells expressed at least these two receptors (**Supplementary Fig. 3A)**.

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The binding affinities of IgA isotypes on different receptors were then compared by thermopheresis with increasing ligand/receptors molar *ratio*. We observed a higher affinity of mIgA2/dIgA2 than dIgA1 and mIgA1 to DC-SIGN **(Fig. 1D)** which is also confirmed by affinity-based ELISA assay (**Suppl. Fig. 2D**) and immunofluorescence. dIgA2 and dIgA1 bound also Dectin-1 with the same affinity **(Fig. 1G and Supplementary Fig. 2C)** which is not confirmed by immunofluorescence and ELISA assay **(Suppl. Fig. 2D),** which could be due to the inherent conformation of the recombinant receptor**.** dIgA1 and to a less extent mIgA1 were able to bind to CD71 **(Fig. 1E).** Affinity for FcαRI was higher for dIgA1 but almost the same for mIgA1 and mIgA2 **(Fig. 1F and Supplementary Fig. 2B)**. Thus, we emphasized a different binding capacity of IgA isotypes to FcαRI, Dectin-1, DC-SIGN and CD71 expressed on moDC.

Dimeric IgA induce an over-expression of their cognate receptors on DC surface.

moDC had been described as comprising two subsets discriminated by the expression of the surface marker CD1a (**Supplementary Figure 3B and 3C**) and displaying different functions [19, 20]. We hypothesized that IgA binding could induce the up-regulation of their receptors. Without any stimulation, moDC constitutively express DC-SIGN, Dectin-1 and a low level of FcαRI but did not express CD71 (**Supplementary Figure 3C**). Only a 2-fold CD71 over-expression on moDC after a treatment with TNF-α, IL-1β and IL-6 was observed (**Fig. 2A**), as previously described [21]. Likewise, treatment of moDC with dimeric or secretory IgA1 and IgA2 significantly increased CD71 and FcαRI expressions by around 1.3-fold (**Fig. 2A and B**). In addition, incubation of moDC with all the IgA isotypes and isoforms except mIgA2 led to a 1.5-fold increase of DC-SIGN and Dectin-1 expression (**Fig. 2C and D**). Thus, we pointed out the capacity of dimeric IgA1 and IgA2 to increase the expression of their receptors, especially DC-SIGN and Dectin-1. This effect was more pronounced on CD1a- moDCs (mDC2) with the IgA2 (**Supplementary Fig. 3B**).

Dimeric IgA efficiently mature moDC .

Since both IgA isotypes and isoforms differently interact with receptors on moDC, we wondered whether they also differently stimulated moDC. To avoid a potential effect of endotoxins that could be present in our IgA preparations, experiments were performed in the presence of polymyxin B. In this case, a conserved ability of moDC to be activated with a cytokine cocktail was observed (**Fig. 3A-D**). Expression of CD80 was not affected by incubation with any IgA isotype or isoform (**Fig. 3A**). mIgA had a limited effect on the expression of co-stimulatory molecules on moDC. In contrast, expression of CD40 was increased by 1.2-folds by dIgA1 and SIgA1 and by 1.5-folds with dIgA2 and SIgA2 (**Fig. 3B**). In addition, dIgA1 induced a 1.3-folds increase of CD86 and HLA-DR up to 2.1-fold with dIgA2 (**Fig. 3C and D**).Thus, moDC seem to be more efficiently activated by dimeric IgA isoforms, especially dIgA2 (**Supplementary Table 1**). The same ability of dimeric IgA2 to upregulate

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CD86 and HLA-DR were also observed on the activation of human mucosal $CD1a^+$ or $CD1a^-$ DCs purified from Crohn's patient gut biopsies **(Supplementary Fig. 4A-C).**

dIgA2 and dIgA1 induce different inflammatory profiles in moDC

In order to characterize the immune profile triggered by monomeric, dimeric or secretory IgA1 and IgA2, we performed a transcriptomic analysis of IgA-treated moDC **(Fig. 4A-C)**. mIgA1 increased the expression of genes involved both in innate immune cells attraction such as chemokine (C-C motif) ligand 16 (CCL16), chemokine (C-X-C motif) ligand 1 CXCL1, CXCL10 and in immune cell maturation/activation such as tumor necrosis factor ligand superfamily (TNF_{SF11}) (**Fig. 4A**). In contrast, mIgA2 decreased expression of CXCL1, CXCL10 and TNF_{SF11}. In addition, 14 other genes involved in cell attraction such as CCL19, CXCL8, CXCL2 or CCL5 or in immune cell activation such as CSF2, RELB, IL-12A/B were down-regulated more than 2-folds (**Fig. 4A**). Gene expression profiles after dIgA and SIgA activation were quite similar, suggesting a low impact of the secretory component (**Fig. 4B-C**). 16 genes over-expressed by mIgA1 were down-regulated by dIgA1 as CCL16, IFNγ, CD40L and TNF_{SF11} but also genes involved in pathogens recognition (TLR9 or TLR7), in angiogenesis (CXCL12) and in monocytes and T cell recruitment (CCL11, CCL8 and CCL13)(**Fig. 4A-B**). dIgA1 also decreased expression of genes involved in immune cell maturation and activation (CLEC4C, IL-2, IL-12 A/B and CSF2) (**Fig. 4A-B**). The most mIgA2 down-regulated genes were significantly up-regulated by dIgA2 (TNFα, CD40L, IFNγ and IL-12A/B) (**Fig. 4A-B**). In particular, neutrophils, macrophages and T cell chemoattractant and maturating coding genes such as CCL19, CXCL8, CXCL1 and CXCL2 and CSF2 were over-expressed (**Fig. 4A-B**). All together, these results indicate that IgA isotypes but also isoforms induce different inflammatory profiles of gene expression in moDC. No or low influence of SC in DC activation has been observed (**Fig. 4C**). In order to get further insights into the effect of IgA interaction with moDC, cytokine production was measured in the moDC culture supernatants after IgA stimulation **(Fig. 4D-F)**. Similar effects on cytokine secretion were observed between dimeric and secretory IgA **(Fig. 4D-E)**. In contrast, the dimeric isoforms were much more potent than the monomers to stimulate cytokine production by moDC. TNF-α and IL-6 levels were increased 5-fold and 10-fold by dIgA1 (**Fig. 4D**) and 33-fold and 700-fold by dIgA2, respectively (**Fig. 4E**). In addition, an increase of around 1.5-fold of IL-16 was observed with dIgA1 and IL-23 secretion was increased by 4-fold after stimulation with dIgA2 (**Fig. 4D-E**). Dimeric IgA also induced a concomitant production of the anti-inflammatory cytokine IL-10, which was increased by 1.6-folds with dIgA1 (**Fig. 4D**) or 7-folds with dIgA2 (**Fig. 4E**). Although a similar secretion of the T cell chemoattractant IL-16 was observed between dIgA1 and dIgA2 stimulation (**Fig. 4F**), dIgA2 induced a higher secretion of pro-inflammatory cytokines such as TNF-α (~80-fold), IL-6 (~2-fold) and IL-12p40 (~20-fold) combined with IL-23 (~5-fold) than dIgA1 (**Fig. 4F**).

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Our results indicate a stronger effect of dIgA on gene transcription and cytokine secretion by DC compared to mIgA. In addition, the effect of dIgA2 was stronger than dIgA1 and skewed towards a pro-inflammatory profile.

IgA2 induce a strong activation of cytotoxic CD8 T lymphocytes

To assess the quality of DC conditioning with IgA, we first compared the ability of mIgA1 and mIgA2 to induce T cell proliferation in a mixed lymphocyte reaction (MLR). No increase in the proportion of activated $CD25^+$ $CD4^+$ T cell was observed in the presence of moDC after mIgA1 or mIgA2 stimulation (**Fig. 5A**). Interestingly, whereas only a limited effect was induced by mIgA1, a significant 1.4-fold increase of activated $CD25^+CD8^+$ T cell proportion was observed after mIgA2 stimulation (**Fig. 5B**). We then used a human model of *in vitro* T cell priming to test the influence of mIgA1 and mIgA2 on the activation of specific effector CD8 T cells from naïve T cells [22]. Melan-A-specific CD8 T cells were primed in the presence of both mIgA1 and mIgA2 (**Fig. 5C, Supplementary Fig. 5**). Interestingly, the presence of mIgA2, but not mIgA1, yielded a higher expression of the transcription factor T-bet (**Fig. 5D, Supplementary Figure 5)** within primed T cells, which was not the case for Eomes (**Fig. 5E**). Increased expression of granzyme B and perforin in Melan-A specific T cells was observed only with mIgA2 (**Fig. 5F-G, Supplementary Fig. 5**). Preliminary data suggest that the effect of IgA2 was enhanced with its dimeric form (**Supplementary Fig. 5)**. Taken together, these findings show that both monomeric IgA1 and IgA2 can promote the induction of antigen-specific CD8 T cell responses, but with a highest potential and a Th1 *bias* for IgA2.

Discussion

In this study, we aimed to clarify the ability of different IgA isotypes and isoforms to interact with their putative receptors expressed on moDC and their related immune effector functions. We have found that IgA1 preferentially bind to FcαRI whereas the affinity of IgA2 is higher for DC-SIGN and that their respective affinities are increased with the dimerization. We also highlighted that dimeric IgA, modulate the expression of their own receptors on moDC. In addition, we demonstrated that dIgA stimulate DC, either by down regulated inflammatory genes with dIgA1 or by increasing expression and secretion of pro-inflammatory molecules with dIgA2. Such immune profiles may engage various pathways leading to the observed priming of functional cytotoxic $CD8^+$ T cells. "Classical" moDC called mDC1 were described as CD1a expressing cells, secreting a high amount of IL-12p70 and inducing a Th1 differentiation [23]. However, a study described the presence of a functionally distinct cell subset, called mDC2 cells, which did not express the CD1a marker [19]. Our study further describes differences between mDC1 and mDC2 [19, 20] such as diverse expression profiles of IgA receptors at steady state and differential responses to interaction with IgA isotypes and isoforms. Indeed, expression of surface activation markers was modulated only on mDC2 after a

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mIgA stimulus. Interestingly, stimulation of mDC2 with LPS led to an opposite effect, suggesting that this subset uses different pathways to respond to mIgA or LPS. Functional studies of individual mDC1 or mDC2 subsets would specify more precisely their role in the modulation of immunity in response to interaction with IgA. In addition, the FcαRI, the transferrin receptor (CD71) and the DC-specific ICAM-3 grabbing nonintegrin (DC-SIGN) were found to be expressed on blood myeloid cells and on moDC [24] [21] [25]. We similarly demonstrate that Dectin-1 is expressed on these cells. FcαRI is a transmembrane receptor which interacts with the Fc domain of both IgA1 and IgA2, with a higher avidity for immune complexes than monomeric or dimeric IgA. The presence of alternative IgA receptors was suggested by addition of anti-FcαRI antibody on moDC which did not abrogate IgA binding [26]. CD71 was described as an IgA1 specific receptor, with a higher avidity for monomeric IgA on Daudi cells [8]. Alternatively, since incubation with mannose decreased SIgA binding on moDC, competition for C-type lectin receptors which bind glycans was suspected. Thus, both DC-SIGN on Chinese hamster ovary (CHO-S) or THP-1 cells and Dectin-1 on intestinal epithelial cells were shown to specifically bind SIgA [9, 10]. Our study demonstrates a higher affinity of IgA1, especially the dimeric form for CD71. We also show here the ability of DC-SIGN and Dectin-1 to interact with mIgA2/dIgA2. Thus, our results suggest a potential regulatory function of IgA that depends more particularly on the expression of DC-SIGN and Dectin-1 on DC. Since DC-SIGN (i) is highly expressed on moDC (ii) preferentially binds IgA2, especially dIgA2 and (iii) its expression is increased by dIgA2, one could hypothesize that these processes contribute to the stronger DC activation and the subsequent CD8 T cell induction observed with dIgA2. As DC-SIGN is involved in DC interaction with T cells *via* ICAM3 [25], it's up-regulation by dIgA2 could play a role in CD8 T cell activation. Based on our observations, it would be interesting to study the pro- or anti-inflammatory effect of IgA molecules in relationship to receptor expression in various cells and tissues as for example in mucosa. Except their role in IgA nephropathy [27], serum monomeric IgA1 were thought to play a minor role in immunity whereas both the proportion and the function of monomeric IgA2 remain to be clarified. Monomeric IgA induce anti-inflammatory response in absence of antigen *via* the FcαRI [28], for example by inducing death of activated neutrophils [29]. A role for other receptors has been suspected since inhibition of Th17 cell response by mIgA occurred independently of FcαRI and DC-SIGN [30]. Our results demonstrate a slight induction of chemoattracting and maturing molecules response induced by mIgA1 with (i) down-regulation of the CD86 co-stimulatory molecule and (ii) absence of pro-inflammatory genes up-regulation and limited production of cytokine. Interestingly, we also demonstrate a mIgA1/CTL pathway with induction of (i) CCL8 and IL-16 expression, two T cell chemo-attractants [31], (ii) proteins involved in T cell activation such as CD40 or TAPBP, a molecule involved in MHC-I processing [32], and (iii) a mIgA1-dependent cytotoxic CD8 T cell

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activation. In this context, further investigations focusing on a potent regulatory role of this cell population, *e.g.* involving killing pro-inflammatory cells, are worth to pursue.

One important function of dIgA is to impede inflammation. This process is mediated either by immune exclusion or by direct effects such as induction of tolerogenic DC *via* SIgA binding on DC-SIGN [33]. Interestingly, we demonstrate that dIgA1 amplify the anti-inflammatory effect of mIgA1 on moDC. Indeed, dIgA1 downregulate pro-inflammatory genes in moDC and trigger a low diversity of cytokines secreted in limited quantity compared to dIgA2 except for IL-16 and IL-21. Besides, some studies showed anti-inflammatory properties of TNF-α and IL-6 depending on their quantities and timing of expression [34, 35] [36]. In addition, the high secretion of IL-12p40 in absence of IL-12p35 or p19 counterparts to form respectively IL-12p70 or IL-23 [37] could indicate the formation of the IL-12p80 homodimer, considered as a CD8 T cell or DC chemoattractant [38]. Since IgA1 may also induce T cell recruitment *via* CCL5 and IL-16 chemoattractant and promote CD8 T cell response, investigating the involvement of T cell in anti-inflammatory response could be relevant. DIgA are also involved in pro-inflammatory mechanisms, for example through the recruitment and activation of neutrophils in mucosa [12, 13]. Besides, immune complexes could modulate the inflammatory effect described above. Binding of antigens to mIgA1 forms immune complexes which are internalized by mature and immature DC *via* FcαRI to induce a pro-inflammatory response [18, 21]. It would be interesting to investigate the type and amplitude of immune response induced by dIgA1 bound to their specific Ag to observe whether the same pro-inflammatory response as mIgA1 takes place. This concept would be in adequacy with the dual function described for mucosal dIgA. Indeed, interaction of IgA with commensal bacteria induce tolerance whereas interaction with pathogens induce either immune exclusion or pro-inflammatory response with recruitment of immune cells such as neutrophils [39] [12]. In addition, an alternative observation related to the dual role of IgA in the intestine is linked to the T cell-dependent (TD) or independent (TI) induction of IgA production. Indeed, it is suggested that TD response induce high affinity IgA responsible for the pro-inflammatory response to pathogens whereas low affinity IgA induced by a TI pathway bind microbiota or inoffensive elements to trigger immune tolerance [39]. In this context, our results would require more investigation to determine whether the TI-induced IgA are mostly IgA1 and TD-induced IgA mostly IgA2. A second explanation of the dual role of dIgA could be that dIgA1 would be in charge of anti-inflammatory responses whereas dIgA2 would induce a pro-inflammatory response. Indeed, dIgA2 induce a clear pro-inflammatory response regarding the gene expression and the cytokine secretion profiles of moDC. Proteins involved in the inflammatory NF-κB pathway such as RELB or NFKB1 are over-expressed and TNF-α and IL-6 are highly produced by DC. In parallel, IL-12p40, which is a subunit of IL-23, is highly secreted together with IL-23, indicating a potential role of

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in inducing a Th17 response after moDC activation by dIgA2 [40]. This result is in accordance with the Th17/IgA axis recently highlighted [41]. Moreover, recruitment of neutrophils by IgA in the intestine is well documented [12, 13]. In accordance with this finding, we have observed that dIgA2 induce over-expression of neutrophil chemoattractants by moDC such as CXCL8, CXCL1, CXCL2 and CCL3. Here, we pointed out a limited role played by secretory component (SC) in the interaction of SIgA with moDC and their activation. We may hypothesize that SC does not interfere with the effects triggered by dIgA, but could be required for immune exclusion [42] and to stabilize dIgA in the intestinal environment [43]. Our preliminary data demonstrate that when mIgA1 and mIgA2 were used to stimulate human mucosal $CD11c^+$ DC extracted from human biopsies, similar results than with moDC were observed (**Supplementary Figure S3**). We need to determine which mucosal DC subsets are implicated in the interaction with IgA and how dimeric or secretory IgA act on such cells. Several DC populations have been described in intestinal mucosa such as the $CD103^+$ DC, thought to be involved in Ag presentation in mesenteric lymph node [44], or the $CX3CR1^+$ DC, thought to be involved in Ag capture in the *lamina propria* [45]. The expression of IgA receptors on these populations, together with their inflammatory profile in response to IgA stimulation should be explored. The functionality of CTLs such as the intraepithelial CD8 αα [46], as well as the non-conventional intestinal T cells such as the Mucosal-Associated Invariant T cells (MAIT) in the gut should also be considered to understand the impact of IgA isotypes and isoforms. Indeed, supernatant of activated MAIT cells induces the production of IgA by B cells [47].

We observed significant DCs activation by dimeric IgA2 isoforms compared to monomeric IgA1/2 or dimeric IgA1. This mechanism could be explained by a different ability (affinity or avidity) of binding to different receptors but also to the same receptors. Indeed, affinities of mIgA isoforms with their receptors are quite weak and similar, suggesting a low activation signaling in downstream. The ability of the different IgAs to bind to multiple receptors at the same time is also an important issue. The activation process may require a threshold of linked receptors as demonstrated for example for the TCR cross-linking. The transcriptomic signature is clearly different between different isotypes and isoforms so the activation/inhibition process is also different. Another important issue/possibility could be the different glycosylation pattern of IgAs which could first explain the different affinities/avidities observed to the different receptors but could be also implicated in the binding to other sugar-specific receptors. Finally, dIgA2 activation is closely related to DC-SIGN, Dectin-1 and to a lesser extent to FcRα, since they are the main IgA2-binding receptors. The simultaneous cross-linking of these three receptors in moDC could lead to high $CD8^+$ T cell activation. Our hypothesis would require investigating the relationship between IgA isoforms/isotypes and their receptors, the cross-binding and the downstream pathways.

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We have demonstrated that while IgA can bind to different receptors displayed on moDC, dIgA1 present a better affinity for FcαRI and dIgA2 for DC-SIGN. Our data also highlight that dIgA1 induce an exacerbation of the anti-inflammatory response induced by mIgA1 in the absence of antigen. In contrast, we showed a strong pro-inflammatory response induced by moDC activated with dIgA2. Interestingly, both processes lead to activation of CD8 T cells. We have also emphasized a modulatory role of IgA which induced the overexpression of their own receptors on DC. Here we show the relationship between IgA isotypes and isoforms and their functionality in the control of immune responses. The ability of the different IgA to stimulate/regulate mucosal immunity should be considered in physiopathological conditions such as Inflammatory Bowel Diseases, IgA nephropathy or coeliac disease [27, 48]. Our results are also of importance for the use of IgA to serve as mucosal vaccine vector [49, 50].

Materials and methods

Flow cytometry. Cell staining was performed in a buffer containing PBS, FBS (2%) and EDTA (1mM). Dead cell exclusion was performed with 7AAD (BD Biosciences, Le Pont de Claix, France) or with a LIVE/DEAD™-488nm staining (Invitrogen, Toulouse, France) for MLR experiment. Staining antibodies were diluted at 1/200 except specified and cell staining was analyzed with a BD FACS Canto II cytometer (BD Biosciences). The following antibodies were used for studying the IgA-receptor profile of human moDC: Anti-Fc□RI BV510 (BD Biosciences), anti-CD71 FITC (BD Biosciences), anti-Dectin-1 PE (BD Biosciences), anti-DC-SIGN BV421 (BD Biosciences), FITC mouse IgG2a, κ isotype control (BD Biosciences), BV421 mouse IgG1, κ isotype control (BD Biosciences), PE mouse IgG2a, κ isotype control (BD Biosciences), BV510 mouse IgG1, κ isotype control (BD Biosciences). The following antibodies were used for testing maturation and phenotype of human moDC: anti-DC-SIGN/DC-SIGN BV421 (BD Biosciences), anti-CD1a APC-Vio770 (Miltenyi Biotec, Paris France), anti-CD14 PE (Miltenyi Biotec), anti-CD11b PE-Vio770 (Miltenyi Biotec), anti-HLA-DR BV510 (BD Biosciences), anti-CD80-PE-Vio770 (Miltenyi Biotec), anti-CD86-FITC (Miltenyi Biotec), anti-CD40-PE (Miltenyi Biotec). The following antibodies were used for testing maturation and phenotype of DC from human gut: anti-CD68 FITC (BD Biosciences), anti-CD1c PE (BD Biosciences), anti-CD11b APC (BD Biosciences), anti-CD11 PE-Cy7 (BD Biosciences), anti-CD103 BV421 (BD Biosciences), anti-CX3CR1 BV510 (BD Biosciences), anti-HLA-DR APC-H7 (BD Biosciences), anti-CD80 APC (Biolegend, San Diego, USA). The following antibodies were used for testing proliferation and activation of human T cells after MLR: anti-CD3 BV510 (BD Biosciences), anti-CD8 PE-Cy7 (BD Biosciences), anti CD4 APC (BD Biosciences), anti-CD25 PE (BD Biosciences), anti-Ki67 BV421 (BD Biosciences). We followed the “Guidelines for the use of flow cytometry and cell sorting in immunological studies” [51].

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Human IgA. Endotoxin-free human monomeric anti-hCD20 mIgA1 and mIgA2 were produced in CHO cells and purified by affinity chromatography with peptide M. (Invivogen, Toulouse, France). The dimeric dIgA1 (Nordic Mubio, Susteren, Netherland) were mAb taken from a patient myeloma with a tested purity around 98% or was purified from IgA1 hybridomas (clone BT1 and BT2 immortalized from plasma cells taken from the *lamina propria* of a Crohn's disease patient. The dimeric dIgA2 were mAb obtained from different IgA2 hybridomas (clone HF2, HF3 and HF4) immortalized from plasma cells taken from the *lamina propria* of a Crohn's disease patient (DDXK-HuBBB, Dendritics Lyon, France). Clones BT1, BT2, HF2, HF3 & HF4 supernatants were concentrated in 100K Amicon (Millipore, France), filtered in 0.22µm and purified by affinity chromatography with peptide M agarose (InvivoGen). Secretory IgA (SIgA) were formed by combining equimolar quantity of dimeric IgA and secretory component [52, 53]. The purity of each isoform was tested by Western Blot (higher than 85%). For all *in vitro* experiments, polymyxin B was added.

Measure of IgA/receptors affinity by ELISA. 96-well maxisorp plates (ThermoFisher) were coated O/N with either the FcαRI or the DECTIN-1 receptors-Fc (R&D systems, Lille, France) or with IgA (for the DC-SIGN and CD71 ELISA). FcαRI and Dectin-1 concentrations began at $6x10^{-7}$ mol/L and serial dilutions were performed at 1/3 whereas IgA concentration was stable at $6x10^{-8}$ mol/L in PBS. After 2 hours of blocking (PBS, tween 0.05%, BSA 1%), the plates were washed three times with PBS/Tween (0.05%). Either the IgA ($6x10^{-8}$ mol/L) or the DC-SIGN or CD71 (serial dilutions beginning at $4.8x10^{-8}$ mol/L) receptors-Fc were added and incubated during 2h. After three washes, plates were incubated either with an anti-human IgA HRP (Sigma-Aldrich) or with an anti-human DC-SIGN (Life technologies**, Illkirch, France**) or with an anti-human CD71 antibody (Life technologies). After three washes, plates previously incubated with the anti-human DC-SIGN or CD71 were incubated with anti-rabbit IgG coupled to horseradish peroxidase (HRP) (Cell signaling technology). Plates were eventually incubated with TMB (Tebu-bio laboratories) after 4 last washes during 30min maximum and the reaction was stopped with hydrochloric acid. The optical density (OD) was read at 450nm (TECAN).

Microscale Thermophoresis. Recombinant receptors FcαRI (R&D systems, Lille, France), Dectin-1 (R&D systems), DC-SIGN (R&D systems) and the human transferrin receptor CD71 (R&D systems) display a N-terminal histidine tag. Coupling of receptor (200nM) with the RED NT-647 (100nM) was performed using « Monolith NT™ His-Tag Labeling Kit RED-tris-NTA » (NanoTemper technologies, München, Germany) following the manufacturer's protocol. Labeled receptors at 25nM final were added separately to a range of human dimeric/monomeric IgA1 or IgA2 2-fold dilutions from 25µM to 0,3nM in PBS, Tween 0.05%, then loaded in standard capillaries (NanoTemper) for MST

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measurement. The strength of receptor/ligand interaction was measured from the diffusion of the labeled molecule in function of ligand concentration when a temperature gradient was applied on the capillaries, using a thermophoresis reader (Monolith NT.115, NanoTemper). K_D was calculated from the titration curves using the MO.Affinity Analysis Software (NanoTemper).

Monocyte derived dendritic cells (moDC) preparation. Human PBMCs were obtained from buffy coats of healthy volunteers (EFS Auvergne-Rhône-Alpes) by density gradient centrifugation over a lymphocyte separation medium (Eurobio; Abcys, Courtaboeuf France). Monocytes ($CD14^+$) were positively sorted from PBMC with magnetic beads according to manufacturer's instructions (Miltenyi Biotec, Paris France). Differentiation of moDC was induced by culture of monocytes with complete RPMI medium (RPMI 1640, 10% FBS, 1% penicilline/streptomycine) supplemented with 100ng/mL of huGM-CSF (Miltenyi Biotec) and 50ng/mL of huIL-4 (Miltenyi Biotec) for 6 days with addition of fresh medium at day 3. The monocyte differentiation into moDC ($CD14^-$, $CD11b^+$, DC-$SIGN^+$ and $CD207^-$) was checked by flow cytometry.

moDC maturation. moDC were cultured at $1x10^6$ cells/mL in 24 wells plates in complete RPMI medium. A treatment with 50µg/mL of polymyxin B (InvivoGen) was done except for comparison between mIgA1 and mIgA2 (Fig. 2 and 3). Reagents were then added at indicated concentrations for moDC stimulation during 24hrs: 500ng/mL of Lipopolysaccharides from E. coli (Sigma), cytokines activator cocktail composed by 25ng/mL of human IL-1β (Miltenyi), 100ng/mL of human IL-6 (Miltenyi) and 50ng/mL of human Tumor Necrosis Factor (TNF)-α (Miltenyi), the various IgA at 12µg/mL.

moDC immunostaining for confocal microscopy. LabTek® slides (Sigma-Aldrich, St Quentin Fallavier, France) were treated with 500µL of Poly-L-lysine (Sigma) and incubated 1hr at 37°C. Slides were washed in PBS and dried 1-2hrs under a safety cabinet. $1x10^6$ moDC/compartment were seeded in complete RPMI medium O/N at 37°C. After 2 gently washes with PBS, cells were incubated with 6µg of antibodies in 200µL of complete RPMI at 37°C during 1hr. After 2 PBS washes, cells were fixed 5min (PBS, 4% formaldehyde), washed again and non-specific sites were blocked (PBS, BSA 1%) during 15min at RT. Cells were permeabilized (PBS, BSA 1%, Triton 0.1%) 2min and washed 2 times with PBS. Primary Ab were added 1hr at 4°C : 5µg/mL of rabbit anti-human CD71 (Thermofisher, Illkirch, France), 20µg/mL of rabbit anti-human DC-SIGN (Thermofisher), 5µg/mL of rabbit anti-human Dectin-1 (Thermofisher), rabbit anti-human FcαRI (1/500, Abcam, Paris, France), 5µg/mL of goat anti-human IgA Alexa555 (Southern Biotech, Birmingham, USA). Cells were washed 2 times with PBS and incubated 45min at 4°C with 5µg/mL of the secondary antibody goat anti-rabbit IgG Alexa 488 (Southern Biotech). Cells were washed with PBS and the nucleus was stained 5-10 min with

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Draq5 (1/1000, Abcam). After 2 washes, cells were mounted between slide and slip cover with vectashield (Vector laboratories) and eventually observed with the FLUOVIEW FV1200 laser scanning confocal microscope at RT (Olympus IX83, Tokyo, Japan) and equipped with the FV10-ASW4.1 imaging software (Olympus, Hamburg, Germany). Image processing was performed with ImageJ Software.

Expression profile by qRT-PCR. RNA from frozen pellets of 1-5 x10^5 activated moDC were extracted thanks to a Quick-RNA MicroPrep Kit (Zymo Research). Total RNA quantification was performed with a 2000c nanodrop (Thermos scientific). RNA quality was assessed (Biorad, 700-7103) with the Experion™ system (Biorad, Marnes-La-Coquette, France). Quantification of a RNA panel described in Suppl. Fig 4 was done with Qiagen kits for Human dendritic cells (330404, 330523, 330231) and the Applied Biosystems® 7500 system. qRT-PCR results were analyzed with the data Analysis Center of Qiagen following supplier instructions.

Cytokine and chemokines titration. The evaluation of multiple cytokines/chemokines was performed with a Luminex 100 instrument (Luminex, Austin, Tex) in combination with the personalized Bio-Plex human cytokine panel composed by IL-12p40, IL-12p70, IL-2, IL-4, IL-6, IL-10, IL-21, IL-23 and TGF-β, IFN-α and IFN-β for monomeric forms or IFN-γ, IL-16 (Bio-Rad Laboratories, Hercules, Calif). Dosages were performed as specified by the manufacturer on pure supernatant of activated moDC previously frozen. IL-16 secretion in supernatant was determined by ELISA (Abcam, Paris, France).

***In vitro* priming of antigen-specific CD8$^+$ T-cell precursors.** Naïve precursors specific for the HLA-A2-restricted Melan-A epitope ELA (ELAGIGILTV; Melan-A/MART-1 residues 26–35A27L) were primed *in vitro* as previously published [22]. Briefly, thawed PBMCs were resuspended in AIM medium, supplemented with FLT3L (50 ng/ml; R&D Systems), and plated out at 2.5×10^6 cells/well in a 48-well tissue culture plate. After 24 hours, the ELA-20 peptide was added (1 µM) and the maturation of resident dendritic cells was induced using a cocktail of cytokines including TNF (1000 U/mL), IL-1β (10 ng/mL), IL-7 (0.5 ng/mL) and prostaglandin E2 (PGE2; 1 µM) (R&D Systems). Alternatively, maturation of DC was induced using ssRNA40 (0.5 µg/mL; Invivogen) or 12µg of IgA. ELA-specific CD8$^+$ T-cell frequency and phenotype were determined on day 10 by flow cytometry.

Directly conjugated mAbs were purchased from commercial sources as follows: 1) anti–CD8-APC-Cy7, anti–CCR7-PE-Cy7, and anti–granzyme-BV450 (BD Biosciences); 2) anti–CD27-AlexaFluor 700 (BioLegend); 3) anti–CD45RA-PercP Cy5.5, anti–T-bet-Alexa Fluor 647, Eomes PE-eFluor 610, anti–perforin-FITC (eBiosciences). The amine-reactive viability dye Aqua (Life Technologies) was used to

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eliminate dead cells from the analysis. Intracellular staining for T-bet and Eomes was performed using the Transcription Factor Buffer Set (BD Biosciences), according to the manufacturer's instructions. Intracellular staining for granzyme B and perforin was compatible with this procedure. Staining with all other reagents was conducted according to standard protocols. Data were acquired using an LSR Fortessa flow cytometer (BD Biosciences) and analyzed with FlowJo software version 9.3.7 (Tree Star).

Human intestinal biopsies. Ileum and colon biopsies were sampled. After a mechanic digestion, enzymatic digestion was performed with 10 U/mL of collagenase and 1/500 DNase (Sigma Aldrich, St Quentin Fallavier, France) for 20min at 37°C under agitation. Samples were ground in Medicon (BD Biosciences, Erembodegem, Belgium) and 30µm filtered (BD Biosciences) after 3 washes. Cells were centrifuged at 200g for 5min and resuspended into RPMI medium (RPMI 1640, 10% FBS, 1% penicilline/streptomycine, 2mM L-glutamine, 1% non-essential amino acids, 10mM Hepes, 1mM sodium pyruvate).

Mixed lymphocyte reaction (MLR). Negative cells recovered after CD14 sorting of PBMC were cultured in complete RPMI medium with 2,5ng/mL (50 UI/mL) of recombinant human IL-2 (rIL-2, R&D system). The medium was changed every 3 days until the differentiation and activation of moDC. Before incubation with IgA-primed moDC (24h with 12 µg of IgA), cells were assessed to be mostly live T cells ($CD3^+CD8^+$ or $CD3^+CD4^+$) by flow cytometry. In a 96-wells plate, $1x10^5$ activated moDC were cocultured with $5x10^5$ allogenic T cells in complete RPMI medium supplemented with rhuIL-2. A positive control was performed with T cell incubated without moDC in medium supplemented with PHA at 5µg/mL (Sigma Aldrich, L8754). Five days after T cell stimulation, surface CD3, CD4, CD8 and CD25 staining were performed and cells were fixed and permeabilized (BD). A Ki-67 BV421 staining was performed in order to check the proliferation of T cells.

Statistical analysis. All statistical analyses were performed with InStat software (version 5.02;GraphPad Software, La Jolla, Calif). The normality was tested for each data set with both Shapiro-Wilk and Agostino-Pearson tests. When data could be modeled by a normal distribution, the following tests were performed depending on the comparison: Student t-test (two means comparison), one-way ANOVA (multiple mean comparisons) with Bonferroni correction (comparison of all pair of data sets) or Dunnet's correction (comparison to a control group). Alternatively, non-parametric tests were performed: Mann-Whitney test (two means comparison), Kruskal-Wallis (multiple mean comparison) with or without Dunn's correction (comparison of all pair of data sets). In case of comparison of two independent variables, a two-way ANOVA test was done. Results were represented as Mean ± SEM. P values of less than 0.05(*), less than 0.01(**), and less than

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0.001(***) were considered significant. Statistically significant differences between groups are emphasized by bars connecting the relevant columns. If not, the comparison was automatically with the control group (NS).

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Conflict of interest

The authors declare no commercial or financial conflict of interest

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Figure 1. Different binding of IgA isotypes and isoforms to IgA receptors on moDC. (A) Confocal microscopy of IgA receptors on monocytes-derived dendritic cells (moDC) upon treatment with 12µg of mIgA1, mIgA2, dIgA1, dIgA2 (dIgA1 from myeloma & dIgA2 from HF2 hybridoma), SIgA1 or SIgA2 (prepared with dIgA1 from myeloma & dIgA2 from HF2 hybridoma). Surface and intracellular FcαRI, Dectin-1, DC-SIGN and CD71 were stained (green) on moDC after IgA treatment. Colocalizations (yellow) between receptors and IgA (red) were evaluated on live cells stained for their nucleus with Draq 5 (blue). Pictures are representative from two experiments with 3 different donors (total=6). Magnification (x60) Scale bar 10µm. (B) Quantification of binding intensities (Arbitrary Units) of the same IgAs on FcαRI, Dectin-1, DC-SIGN and CD71. Intensity of co-labeling was estimated on 50 different cells per condition (two experiments with 3 donors performed in triplicate, n=6) and compared with anti-IgA alone. Relative binding intensities were compared with a two-way ANOVA with Dunnett's multiple comparison test (*) $p \leq 0.05$, (**) $p \leq 0.01$ and (***) $p \leq 0.001$. Datas are shown as median +/- SD. (C) After 24hrs of incubation with IgA, moDC were separated depending on the number of IgA-receptors expressed on their surface (FcαRI, DC-SIGN, Dectin-1 and CD71) by flow cytometry. Data are from three experiments with 2-3 donors per experiment, performed in triplicate (n=8). Kruskal-Wallis with Dunn's multiple comparison test was performed. Datas are shown as median +/- SD (D-G) Thermopheresis analysis of IgA binding to the different receptors. Histidine-tagged receptors were coupled with a fluorochrome and the delay of fluorescence recovery after warming was measured after binding with several concentrations of monomeric IgA1 (red line), IgA2 (green line) or dIgA1 (purple line) or dIgA2 (blue line) to DC-SIGN (D), CD71 (E), FcαRI (F) and Dectin-1 (G). Measures at each ligand concentration are displayed as mean +/- s.d. of 3 different experiments in duplicate (n=6). KD was calculated from the titration curves using the MO.Affinity Analysis Software (NanoTemper).

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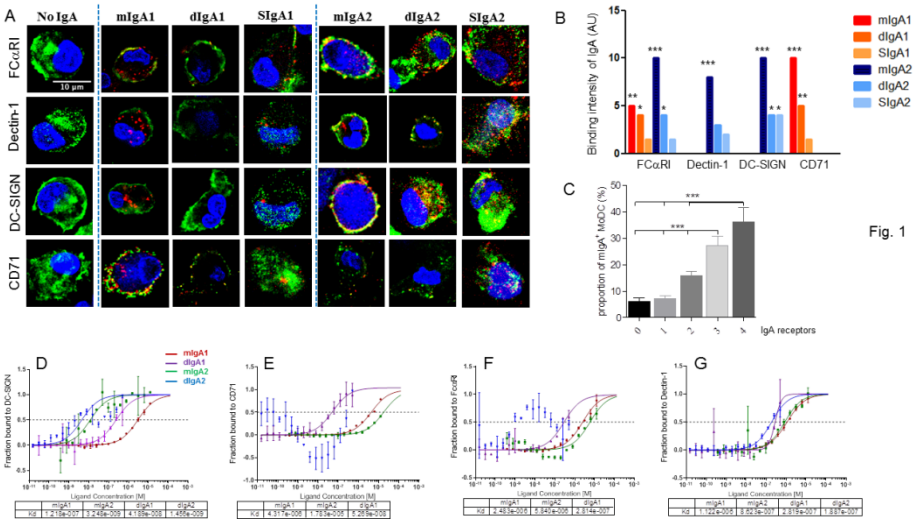

Fig. 1

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Figure 2. Relation between receptors expression on moDC and IgA internalization. (**A-D)** Mean Fluorescence Intensity (MFI) of CD71 **(A)**, FcαRI **(B)**, Dectin-1 **(C)**, DC-SIGN (D) receptor expression on IgA^+ moDCs. Expression was analyzed by flow cytometry after 24hrs with an activation cocktail (Cytokines: IL-1β, IL-6, TNF-α) or IgA1 or IgA2 isoforms. Measures were normalized relative to non-stimulated (NS) moDC to reduce intra-donor biases. Data are obtained from 8 donors and experiments were performed in triplicate. Relative expression means were compared with each other via two-way ANOVA with Dunnett's multiple comparison test. (*) p≤0.05, (**) p≤0.01 and (***) p≤0.001. Data are shown as median +/- SD.

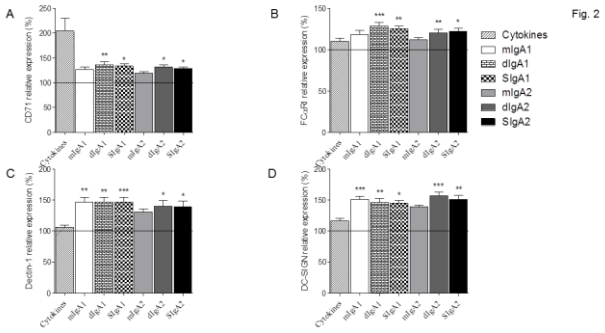

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Figure 3. moDC activation by IgA1 and IgA2 isoforms . (A-D) Flow cytometry analysis of the relative expression of activation markers (CD80, CD40, CD86, HLA-DR) by moDC were obtained by comparing the MFI after 24h of IgA stimulation (12µg/mL) to non-stimulated moDC). A stimulated positive control (Cytokines: IL-1β, IL-6, TNF-α) was used. The increase/decrease of marker expression between them was normalized with unstimulated samples. Data are from five experiments with 3-5 donors per experiment, performed in triplicate (n=19 for IgA1 and 8 for IgA2). Relative expression means were compared with a two-way ANOVA with with Dunnett's multiple comparison test. (*) p≤0.05, (**) p≤0.01 and (***) p≤0.001. Datas are shown as median +/- SD.

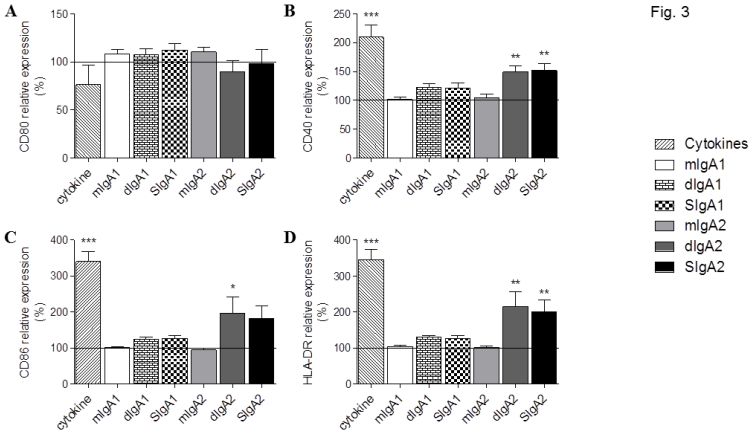

Fig. 3

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Figure 4. Transcriptomic and cytokine profiles of moDC activated by the various IgA isoforms and isotypes. (A-C) RNA expression of moDC from 3 different donors (n=3) stimulated with either mIgA1 or mIgA2 isoforms **(A)**, dIgA1 or dIgA2 isoforms (dIgA1 from myeloma & dIgA2 from HF2 hybridoma) **(B)** or SIgA1 or SIgA2 (prepared with dIgA1 from myeloma & dIgA2 from HF2 hybridoma) **(C).** RNA expression was compared to non-stimulated moDC. Analysis of qRT-PCR results was performed with the help of the Qiagen Data Analysis Center in order to determine the relation between Ct and gene fold-regulation. Genes displaying 2-fold up- or down-regulated expression were considered as affected by IgA stimulation. **(D-F)** Cytokine concentrations in the culture supernatant of moDC stimulated with IgA1 **(D)**, IgA2 **(E)** isoforms or with dIgA1 or dIgA2 **(F)** were determined by Luminex or ELISA assays. A one-way ANOVA with Dunnett's multiple comparison or a Kruskal-Wallis with Dunn's multiple comparison statistical test were used depending on the results of the normality tests (Shapiro-Wilk and Agostino-Pearson). Profiles are shown as radar charts; each axis displays the mean quantity (in pg/mL) of each cytokine/chemokine 24hrs after moDC stimulation. Data are from four experiments with 3-5 donors per experiment, performed in triplicate (n=14). The scale of each axis being different, the values of both minimum and maximum are indicated into brackets [min: max]. (*) p≤0.05, (**) p≤0.01.

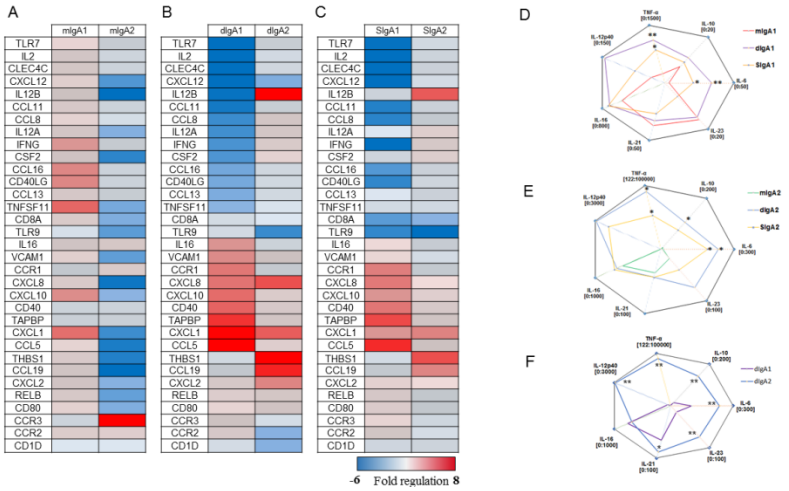

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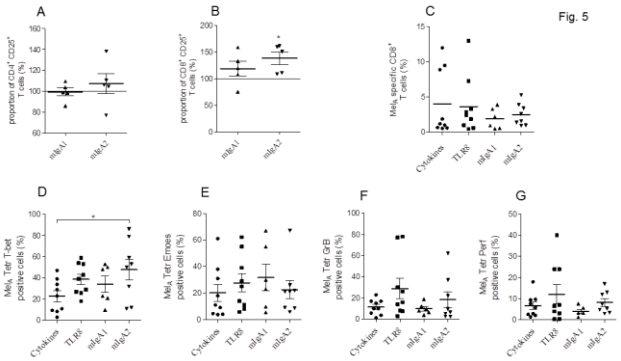
Figure 5. Activation of cytotoxic CD8 T cells in the presence of mIgA1 or mIgA2. **(A, B)** Flow cytometry analysis of the activation of $CD4^+$ **(A)** or $CD8^+$ **(B)** $CD25^+$ T cells in a mixed lymphocyte reaction with moDC primed with mIgA1 or mIgA2 . Non-stimulated moDC were used as the negative control (illustrated by the line at 100%). A Kruskal-Wallis with Dunn's multiple comparison statistical test was performed. Data are from two experiments with 2-3 donors per experiment, performed in duplicate (n=5). **(C-G)** Frequencies of MelanA-specific CD8 T cells **(C)**, and the expression of the transcription factors T-bet (D) and Eomes **(E),** and granzyme B (F) and perforin **(G)** expression were determined by flow cytometry after *in vitro* priming with cytokines (IL-1β, IL-6, TNF-α), TLR8, mIgA1- or mIgA2-stimulated moDC. Data are normalized compared to non-stimulated conditions. We performed a one-way ANOVA with Dunnett's multiple comparison test. Data are from three experiments with 3 donors per experiment, in duplicate (n=9). s.d. are indicated on the graphs; (*) p≤0.05.



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