UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Département de Médecine, CHUV

Generation of reconstituted human plasma-derived secretory-like IgA and IgM and their protective effect on intestinal epithelium

Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

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par

Stéphanie LONGET

Master en Génomique et Biologie expérimentale
de l'Université de Lausanne

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Prof. Renaud DU PASQUIER, Président
Dr. Blaise CORTHESY, Directeur de thèse
Prof. François SPERTINI, Expert
Dr. Laurent FAVRE, Expert

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Generation of reconstituted human plasma-derived secretory-like IgA and IgM and their protective effect on intestinal epithelium

Lausanne, le 17 février 2014

pour Le Doyen
de la Faculté de Biologie et de Médecine

Prof. Renaud Du Pasquier

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"C'est surtout le plaisir de l'inconnu qui me motive" Christophe, chercheur de sons (Le Télégramme, 10.11.13)

Résumé destiné au large public

Les muqueuses sont les membranes tapissant les cavités du corps, tel que le tube digestif, et sont en contact direct avec l'environnement extérieur. Ces surfaces subissent de nombreuses agressions pouvant être provoquées par des agents pathogènes (bactéries, toxines ou virus). Cela étant, les muqueuses sont munies de divers mécanismes de protection dont notamment deux protéines-clés permettant de neutraliser les agents pathogènes : les anticorps ou immunoglobulines sécrétoires A (SIgA) et M (SIgM). Ces anticorps sont, d'une part, fabriqués au niveau de la muqueuse sous forme d'IgA et IgM. Lorsqu'ils sont sécrétés dans l'intestin, ils se lient à une protéine appelée pièce sécrétoire et deviennent ainsi SIgA et SIgM. La présence de la pièce sécrétoire est essentielle pour que les anticorps puissent fonctionner au niveau de la muqueuse. D'autre part, ces anticorps sont également fabriqués dans d'autres parties du corps en général et se retrouvent dans le sang sous forme d'IgA et IgM

Chez l'homme, des thérapies basées sur l'injection d'anticorps donnent de bons résultats depuis de nombreuses années notamment dans le traitement des infections. Bien qu'un certain nombre d'études ont montré le rôle protecteur des anticorps de type IgA et IgM, ceux-ci ne sont que rarement utilisés dans les thérapies actuelles. La principale raison de cette faible utilisation réside dans la production ou la purification des IgA/IgM ou SIgA/SIgM (la forme active au niveau des muqueuses) qui est difficile à réaliser à large échelle. Ainsi, le but de la thèse était (1) d'étudier la possibilité d'employer des IgA et des IgM provenant du sang humain pour générer des SIgA et SIgM et (2) de voir si ces anticorps reconstitués pouvaient neutraliser certains agents pathogènes au niveau des muqueuses.

Tout d'abord, une analyse biochimique des IgA et des IgM issues du sang a été effectuée. Nous avons observé que ces anticorps avaient des caractéristiques similaires aux anticorps naturellement présents au niveau des muqueuses. De plus, nous avons confirmé que ces anticorps pouvaient être associés à une pièce sécrétoire produite en laboratoire pour ainsi donner des SIgA et SIgM reconstituées. Ensuite, la fonctionnalité des anticorps reconstitués a été testée grâce à un modèle de couche unique de cellules intestinales différenciées (monocouches) en laboratoire imitant la paroi de l'intestin. Ces monocouches ont été infectées par une bactérie pathogène, *Shigella flexneri*, responsable de la shigellose, une maladie qui provoque des diarrhées sanglantes chez l'homme. L'infection des monocouches par les bactéries seules ou combinées aux SIgA et SIgM reconstituées a été analysée. Nous avons observé que les dommages des cellules étaient moins importants lorsque les SIgA étaient présentes. Il apparaît que les SIgA neutralisent les bactéries en se fixant dessus, ce qui provoque leur agrégation, et diminuent l'inflammation des cellules. La protection s'est montrée encore plus efficace avec les SIgM. De plus, nous avons vu que les SIgA et SIgM pouvaient diminuer la sécrétion de facteurs nocifs produits par les bactéries.

Utilisant le même modèle des monocouches, la fonctionnalité des IgA issues du sang humain a aussi été testée contre une toxine sécrétée par une bactérie appelée *Clostridium difficile*. Cette bactérie peut être présente naturellement dans l'intestin de personnes saines, cependant elle peut devenir pathogène dans certaines conditions et être à l'origine de diarrhées et d'inflammations de l'intestin via la sécrétion de toxines. Des préparations d'anticorps contenant une certaine proportion de SIgA reconstituées ont amené à une diminution des dommages et de l'inflammation des monocouches causés par la toxine.

L'ensemble de ces résultats prometteurs, montrant que des SIgA et SIgM reconstituées peuvent protéger la paroi de l'intestin des infections bactériennes, nous conduisent à approfondir la recherche sur ces anticorps dans des modèles animaux. L'aboutissement de ce type de recherche permettrait de tester, par la suite, l'efficacité sur l'homme de traitements des infections des muqueuses par injection d'anticorps de type SIgA et SIgM reconstituées.

Résumé

Les muqueuses, telle que la muqueuse gastrointestinale, sont des surfaces constamment exposées à l'environnement et leur protection est garantie par une combinaison de barrières mécaniques, physicochimiques et immunologiques. Parmi les divers mécanismes de protection immunologiques, la réponse humorale spécifique joue un rôle prépondérant et est assurée par les immunoglobulines sécrétoires de type A (SIgA) et M (SIgM).

Les thérapies basées sur l'administration d'IgG apportent d'importants bénéfices dans le domaine de la santé. Bien que des études sur les animaux aient montré que l'administration par voie muqueuse d'IgA polymérique (pIgA) ou SIgA pouvaient protéger des infections, des IgA/SIgA n'ont été utilisées qu'occasionnellement dans les thérapies. De plus, des études précliniques et cliniques ont démontré que l'administration par voie systémique de préparations enrichies en IgM pouvait aussi protéger des infections. Cependant, l'administration par voie muqueuse d'IgM/SIgM purifiées n'a pas été examinée jusqu'à présent. La principale raison est que la purification ou la production des IgA/SIgA et IgM/SIgM est difficile à réaliser à large échelle. Le but de ce travail de thèse était d'examiner la possibilité d'associer des IgA et IgM polyclonales purifiées à partir du plasma humain avec une pièce sécrétoire recombinante humaine afin de générer des SIgA et SIgM reconstituées fonctionnelles.

Tout d'abord, une analyse biochimique des IgA et IgM issues du plasma humain a été effectuée par buvardage de western et chromatographie. Ces molécules avaient des caractéristiques biochimiques similaires à celles des immunoglobulines issues de la muqueuse. L'association entre pIgA ou IgM issues du plasma humain et la pièce sécrétoire recombinante humaine a été confirmée, ainsi que la stoechiométrie 1:1 de l'association. Comme dans les conditions physiologiques, cette association permettait de retarder la dégradation des SIgA et SIgM reconstituées exposées à des protéases intestinales. Ensuite, la fonctionnalité et le mode d'action des IgA et IgM issues du plasma humain, ainsi que des SIgA et SIgM reconstituées, ont été explorés grâce à un modèle *in vitro* de monocouches de cellules intestinales épithéliales polarisées de type Caco-2, qui imite l'épithélium intestinal. Les monocouches ont été infectées par un pathogène entérique, *Shigella flexneri*, seul ou combiné aux immunoglobulines issues du plasma humain ou aux immunoglobulines sécrétoires reconstituées. Bien que les dommages des monocouches aient été retardés par les pIgA et SIgA reconstituées, les IgM et SIgM reconstituées se sont montrées supérieures dans le maintien de l'intégrité des cellules. Une agrégation bactérienne et une diminution de l'inflammation des monocouches ont été observées avec les pIgA et SIgA reconstituées. Ces effets étaient augmentés avec les IgM et SIgM reconstituées. De plus, il s'est révélé que les deux types d'immunoglobulines de type sécrétoire reconstituées agissaient directement sur la virulence des bactéries en réduisant leur sécrétion de facteurs de virulence. La fonctionnalité des IgA issues du plasma humain a aussi été testée contre la toxine A de *Clostridium difficile* grâce au même modèle de monocouches de cellules épithéliales. Nous avons démontré que des préparations enrichies en IgA provenant du plasma humain pouvaient diminuer les dommages et l'inflammation des monocouches induits par la toxine.

L'ensemble de ces résultats démontrent que des IgA et IgM de type sécrétoire peuvent être générées à partir d'IgA et IgM issues du plasma humain en les associant à la pièce sécrétoire et que ces molécules protègent l'épithélium intestinal contre des bactéries pathogènes. Ces molécules pourraient dès lors être testées dans des modèles *in vivo*. Le but final serait de les utiliser chez l'homme à des fins d'immunisation passive dans le traitement de pathologies associées à la muqueuse telles que les infections.

Abstract

Mucosal surfaces, such as gastrointestinal mucosa, are constantly exposed to the external environment and their protection is ensured by a combination of mechanical, physicochemical and immunological barriers. Among the various immunological defense mechanisms, specific humoral mucosal response plays a crucial role and is mediated by secretory immunoglobulins A (SIgA) and M (SIgM).

Immunoglobulin therapy based on the administration of IgG molecules leads important health benefits. Even though animal studies have shown that mucosal application of polymeric IgA (pIgA) or SIgA provided protection against infections, IgA/SIgA have been only used occasionally for therapeutic application. Moreover, preclinical and clinical studies have demonstrated that systemic administration of IgM-enriched preparations could also afford protection against infections. Nevertheless, mucosal application of purified IgM/SIgM has not been examined. The main reason is that the purification or production of IgA/SIgA and IgM/SIgM at large scale is difficult to achieve. The aim of this PhD project was to examine the possibility to associate polyclonal human plasma-derived IgA and IgM with recombinant human secretory component (SC) to generate functional secretory-like IgA and IgM.

First, biochemical analysis of human plasma IgA and IgM was performed by western blotting and chromatography. These molecules exhibited the same biochemical features as mucosa-derived antibodies (Abs). The association between human plasma pIgA or IgM and recombinant human SC was confirmed, as well as the 1:1 stoichiometry of association. Similarly to physiological conditions, this association delayed the degradation of secretory-like IgA or IgM by intestinal proteases. Secondly, the function activity and the mode of action of human plasma IgA and IgM, as well as secretory-like IgA and IgM were explored using an *in vitro* model of polarized intestinal epithelial Caco-2 cell monolayers mimicking intestinal epithelium. Cell monolayers were infected with an enteropathogen, *Shigella flexneri*, alone or in combination to plasma Abs or secretory-like Abs. Even though plasma pIgA and secretory-like IgA resulted in a delay of bacteria-induced damages of cell monolayers, plasma IgM and secretory-like IgM were shown to be superior in maintenance of cell integrity. Polymeric IgA and secretory-like IgA induced bacterial aggregation and decreased cell monolayer inflammation, effects further amplified with IgM and secretory-like IgM. In addition, both secretory-like Abs directly impacted on bacterial virulence leading to a reduction in secretion of virulence factors by bacteria. The functionality of human plasma IgA was also tested against *Clostridium difficile* toxin A using Caco-2 cell monolayers. Human plasma IgA-enriched preparations led to a diminution of cell monolayer damages and a decrease of cellular inflammation induced by the toxin.

The sum of these results demonstrates that secretory-like IgA and IgM can be generated from purified human plasma IgA and IgM associated to SC and that these molecules are functional to protect intestinal epithelium from bacterial infections. These molecules could be now tested using *in vivo* models. The final goal would be to use them by passive immunization in the treatment of mucosa-associated pathologies like infections in humans.

Abbreviations

Ab	Antibody
ADCC	Antibody-dependent cytotoxicity
AID	Activation-induced cytidine deaminase
AJ	Adherens junction
APRIL	A proliferation-inducing ligand
ASGP-R	Asialoglycoprotein receptor
Asn	Asparagin
BAFF	B-cell activating factor
BSA	Bovine serum albumin
CP	Cryptopatch
CSR	Class-switch recombination
Cys	Cystein
DAPI	4’,6-diamidino-2-phenylindole
DC	Dendritic cell
dIgA	Dimeric immunoglobulin A
DM	Desmosome
DMEM	Dulbecco's modified eagle medium
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FAE	Follicle-associated epithelium
FDC	Follicular DC
FCS	Fetal calf serum
FcγR	Fcγ receptor
FcRL	Fc receptor like
FcRn	Neonatal Fc receptor
GALT	Gut-associated lymphoid tissue

GC	Germinal centre
GI	Gastrointestinal
GRO-α	Growth regulated oncogen-α
HCl	Hydrochloric acid
HEV	High endothelial venule
HRP	Horseradish peroxidase
hSC	Human secretory component
i.v	Intravenous
IDC	Interdigitating DC
IEC	Intestinal epithelial cell
IFN	Interferon
IFR	Interfollicular region
Ig	Immunoglobulin
IL	Interleukin
ILF	Isolated lymphoid follicle
Ipa	Invasion plasmid antigen
ITAM	Immunoreceptor tyrosine-based activation motif
IVIg	Intravenous immunoglobulin
J chain	Joining chain
JAM	Junctional adhesion molecule
LPS	Lipopolysaccharide
LSCM	Laser scanning confocal microscopy
M cell	Microfold cell
MCP-1	Monocyte chemoattractant protein-1
mIgA	Monomeric immunoglobulin A
MLN	Mesenteric lymph node
mSC	Mouse secretory component
Mxi	Membrane expression of Ipa
NF-κB	Nuclear factor kappa B

NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
PAR	Partitioning defective adaptor protein
PBS	Phosphate buffered saline
PGN	Peptidoglycan
pIgA	Polymeric immunoglobulin A
pIgR	Polymeric immunoglobulin receptor
PP	Peyer's patch
RT	Room temperature
SC	Secretory component
SCIg	Subcutaneous immunoglobulin
SCR	Secretory component receptor
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SED	Subepithelial dome
SEM	Standard error of the mean
SHP-1	Src homology region 2 domain-containing phosphatase-1
SIgA	Secretory immunoglobulin A
SIgM	Secretory immunoglobulin M
Spa	Surface presentation of Ipa antigen
Syk	Spleen tyrosine kinase
T3SS	Type-III secretion system
TER	Transepithelial eletrical resistance
Tfr	Transferrin receptor
TGF-β	Transforming growth factor-β
Th	T helper
TJ	Tight junction
TNF-α	Tumor necrosis factor-α
UDP	Uridine diphosphate
ZO	Zonula occludens

Units

°C	Degree celsius
g	Gram
h	Hour
kb	Kilobase
kDa	Kilodalton
kg	Kilogram
l	Liter
M	Molar
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
nM	Nanomolar
%	Percent
pg	Picogram
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar

Introduction

1. The gastrointestinal tract

1.1 General overview

The gastrointestinal (GI) tract is a part of digestive system that also comprises associated digestive organs including pancreas, liver, gallbladder and the salivary glands. The GI tract has a surface equal to 400 m^2 and its primary functions are digestion, absorption and assimilation of nutrients[1,2].

The GI tract is subdivided into six major parts: oral cavity, pharynx, esophagus, stomach, small intestine and large intestine. The digestive process begins in the oral cavity. The ingested material is physically broken by chewing and starts to be chemically digested by enzymes found in the saliva. By peristalsis, the material comes through the pharynx and esophagus, then arrives in the **stomach**. At this level, the material is mixed with water and gastric juices containing enzymes to form what it is called chyme. In addition, specialized cells such as parietal, chief and goblet cells take part in digestion process. Parietal cells secrete hydrochloric acid (HCl) that leads to a reduction of pH and allows to denaturate proteins and kill bacteria entered with food. Chief cells secrete pepsinogen that is splitted in pepsin under the effect of HCl and that is involved in protein digestion. Finally, goblet cells secrete mucus forming a protective layer between the gastric mucosa and acidic environment. After the stomach, chyme is delivered into the small intestine. The **small intestine** is formed of a particular structure comprising numerous folds and villi that allows to increase the absorption surface of nutrients. The small intestine is subdivided into three parts: duodenum, jejunum and ileum. The duodenum and the proximal half of the jejunum are the major sites for digestion and absorption of water, nutrients and electrolytes. Intestinal enzymes, but also the pancreatic juice and the bile delivered into the duodenum, take part in digestion. Specifically, the pancreatic enzymes are essential for digestion of fats, proteins and carbohydrates, while the bile is mainly involved in the neutralization of acid and solubilizes fats. After the ileum, the chyme reaches the **large intestine** composed of the cecum, appendix, colon, rectum and anal canal. The large

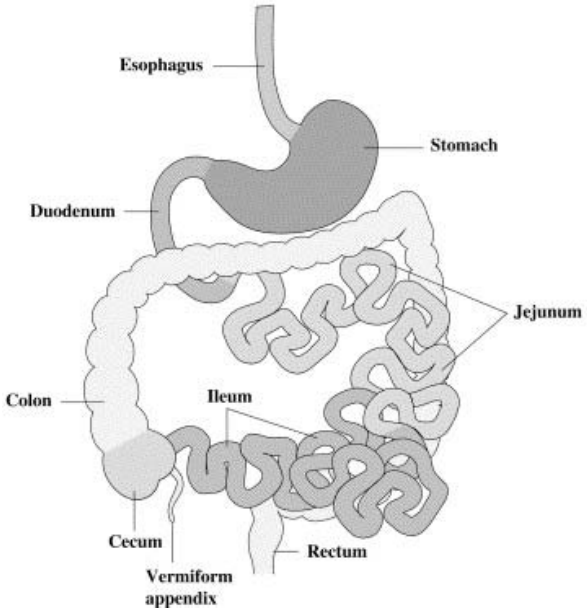

Figure 1: Anatomy of human gastrointestinal tract. The esophagus enters the stomach. The small intestine is divided into three segments: duodenum, jejenum and ileum. The ileum ends up into the large intestine subdivided into the cecum, colon (ascending, transverse, descending and sigmoid colon), rectum and anal canal (adapted from DeSesso and Jacobson, 2001)[3].

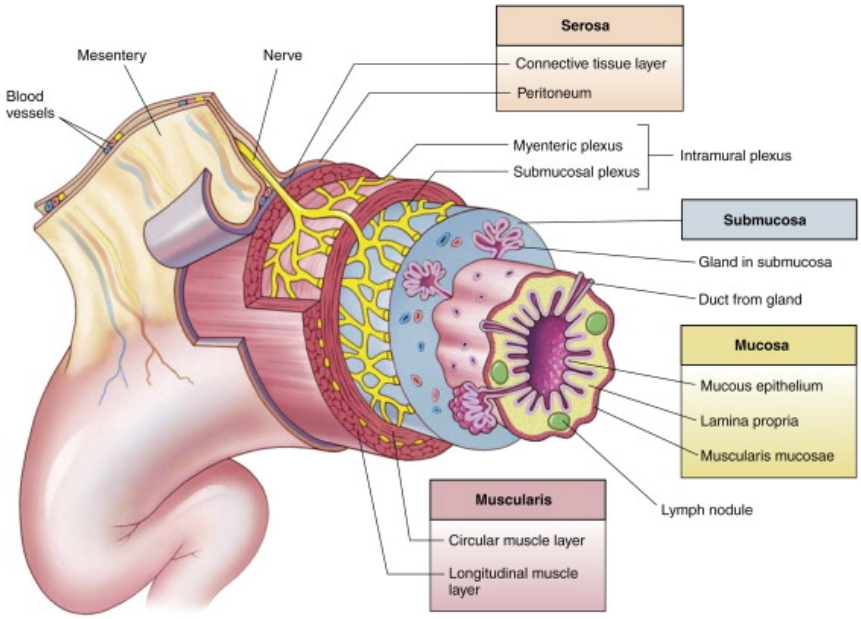

Figure 2: Overview of the wall of gastrointestinal tract. The gastrointestinal tract is composed of four main layers: mucosa, submucosa, muscularis and serosa (adapted from Reed and Wickham, 2009)[2].

intestine predominantly plays an important role in water reabsoption. Moreover, the colon is colonized by numerous commensal bacteria that are crucial to finish metabolizing nutrients. Finally, undigested material is expulsed by the rectum and anus[2,3] (**Figure 1**).

1.2 The intestinal mucosa

The intestinal mucosa is composed of epithelium, lamina propria and muscularis mucosa. The **mucosal epithelium** differs according to the region of the tract. At the level of the oral cavity, esophagus and anal canal, the epithelium consists of stratified squamous epithelial cells. In the stomach, the small intestine and colon, the epithelium is made up of simple columnar or glandular epithelial cells. The **lamina propria** is a connective tissue in which blood and lymphatic vessels are present to support the epithelium and deliver it various nutrients. In addition, the lamina propria plays a role in internalization of luminal digestion products and hormones by the epithelium. Specialized structures called gut-associated lymphoid tissues (GALTs) are also found in the lamina propria. These structures orchestrate intestinal immune responses and will be presented in the section 2 of Introduction. The **muscularis mucosa** is the third sub-layer of the mucosa. It is a layer of smooth muscle that is well developed in the small intestine. Its main role is to give rhythm to million of villi, which promote absorption of chyme (**Figure 2**).

The intestinal mucosa is supported by a connective tissue layer called **submucosa**. It comprises blood and lymphatic vessels, various glands and a nerve network called Meissner's plexus that acts on the **muscularis externa**. This latter comprises a striated muscle in the mouth, pharynx and upper esophagus, whereas in other regions of the tract it is constituted of a smooth muscle circular layer and a longitudinal smooth muscle layer. Auerbach's plexus coordinates the movements of layers allowing the peristalsis of the gastrointestinal tract. Finally, the outermost layer of the tract is the **serosa** that is composed of a connective tissue layer and the peritoneum[2,3,4] (**Figure 2**).

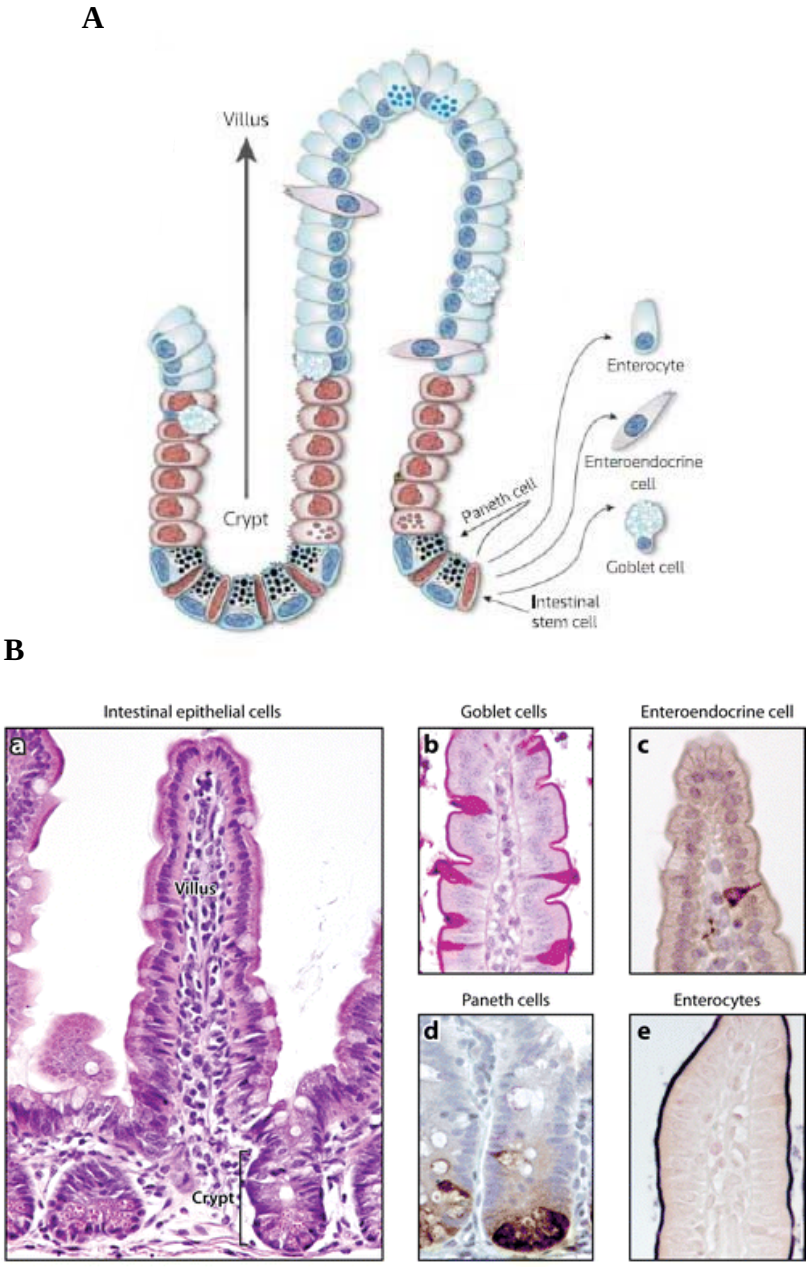

Figure 3: Intestinal epithelium. The intestine is lined by a single layer of epithelial cells organized into crypts and villi. It is constituted of four main cell types: mucus-secreting goblet cells, hormone-secreting enteroendocrine cells, antimicrobial-secreting paneth cells and absorptive enterocytes. These cells differentiate from stem cells found in the crypts. A) The schema shows the organization of the intestinal epithelium and the position of each cell type. B) Hematoxylin and eosin staining shows the morphology of the mouse intestine cells (adapted from Li et al., 2009 and Van Der Flier and Clevers, 2009)[4,5].

1.3 The intestinal epithelium

This part will be deliberately focused on the description of the small intestine's epithelium. This epithelium is organized into crypts and villi and four cell types are found within it: Paneth cells, goblet cells, enteroendocrine cells and enterocytes (**Figures 3 A and B**).

Paneth cells are found in the crypt base. They are composed of granules that contain mainly antimicrobial peptides, lysozymes and defensins crucial to protect from microbial invasion. These cells were also shown to impact on microbiota composition and to play a role in production of vital factors for the stem cells of the small intestine[4,6]. **Goblet cells** are present in the duodenum and the colon. Their proportion among all epithelial cells is close to ca. 4% in the duodenum and ca. 16% in the distal colon. These cells synthesize mucin glycoproteins allowing the formation of an outer and inner mucus layers that provide a protection against damages of epithelium. The outer mucus layer is colonized by commensal bacteria, whereas the bacteria are nearly absent from the inner mucus layer. These cells also secrete trefoil proteins essential for epithelial repair, Resistin-like molecule β (small cystine-rich secreted proteins) with antiparasitic effects and Fc-γ binding proteins leading to a stabilization of the mucin network. Finally, the microflora is shown to modulate goblet cell functions[4,7]. The **enteroendocrine cells** are located within the crypts and villi and represent ca. 1% of epithelial cells. Fifteen subtypes of enteroendocrine cells secrete specific hormones involved in physiological functions such as motility of the gastrointestinal tract. These cells are sensors of luminal contents, mainly nutrients, and transmit chemical signals by exocytosis of hormones to afferent nervous fibres. These cells are also immune sensors and therefore express Toll-like receptors. In addition, a role for enteroendocrine cells in GI repair was also described[4,8]. **Enterocytes** represent more than 80% of epithelial cells and form the basis of the intestinal epithelial barrier. These cells are columnar and polarized with an apical brush-border side. Their main role is the absorption and the transfer of nutrients across the epithelium. Dietary antigens such as amino acids, sugars, ions are transported into the enterocytes either by transcellular transport (receptor-

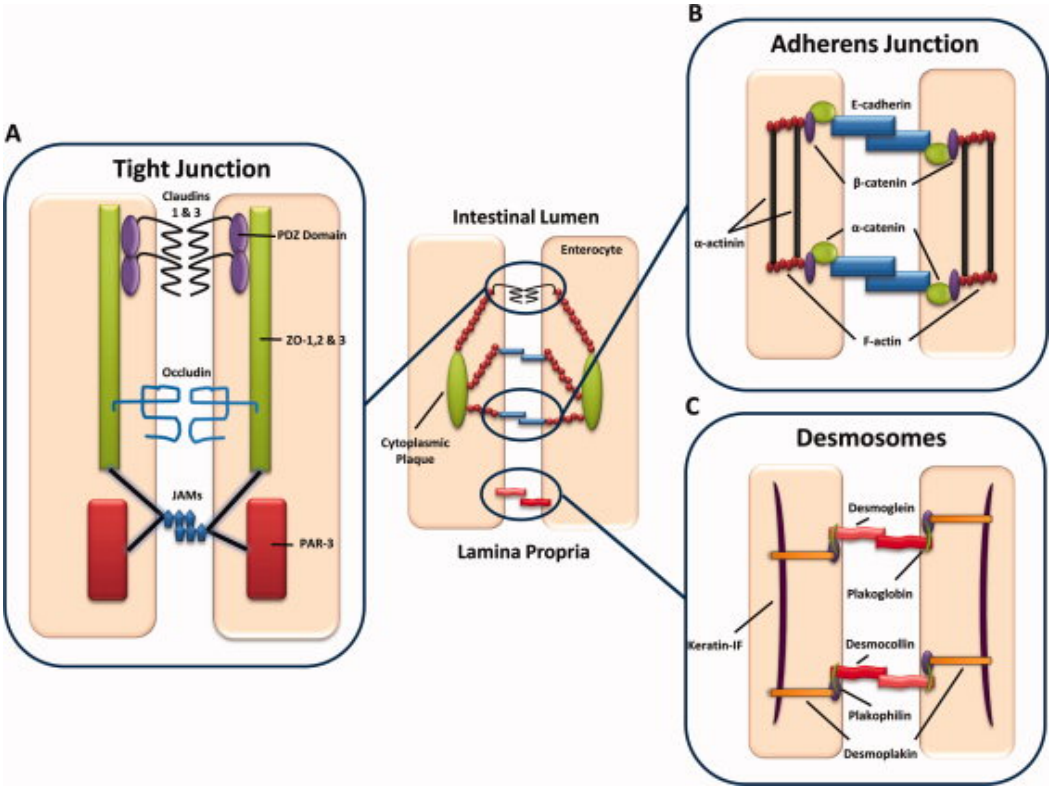

Figure 4: Intercellular junctions interconnecting the intestinal epithelial cells. The three main junctions, from the luminal to the lamina propria side, are the tight junctions (A), the adherens junctions (B) and the desmosomes (C). A. The tight junctions localized at the apical-lateral membrane junction, are composed of transmembrane proteins (claudins, occludin and junctional adhesion molecules (JAMs)) connected to the actin cytoskeleton through the Zonula occludens-1,2,3 proteins (ZO-1,2,3) or partitioning defective adaptor proteins (PARs). B. The adherens junctions consist of cadherin-catenin interactions. C. The desmosomes are anchoring sites for keratin filaments (Adapted from Henderson et al., 2010)[9].

mediated endocytosis or pinocytosis) or by paracellular transport. In the transcellular pathway, the molecules are degraded by enzymes found in the lysosomes, whereas in the paracellular pathway, the molecules are not degraded and are delivered as such into the interstitial space. Enterocytes were also demonstrated to play a role in the antigen presentation to T cells within the epithelium or in the lamina propria[9,10] (**Figure 3 B**).

For the renewal of intestinal cells, progenitor cells are found in the crypts. The stem cells are pluripotent and after a few rounds of cell division, they differentiate and migrate out of the crypts onto the villi[4,11,12] (**Figure 3 A**).

Intercellular junctions

The epithelial cells are connected by intercellular junctions crucial for the maintenance of the intestinal barrier. The three main junctions, from the apical to the basolateral side, are the tight junctions, the adherens junctions and the desmosomes (**Figure 4**).

The **tight junctions** (TJs) are localized at the apical-lateral membrane junction and are composed of more than 50 transmembrane and plaque proteins. Transmembrane proteins are claudins, occludin and junctional adhesion molecules (JAMs). They are divided into tetra-span proteins such as occludin and claudins, and single span proteins such as JAMs. Transmembrane proteins close the space between epithelial cells and mediate the intercellular adhesion. Occludin is especially involved in the regulation of paracellular and intermembrane diffusion of molecules. The presence of an additional protein called tricellulin allows to increase the resistance of the barrier at the level of junctions of three epithelial cells. The claudins play a role of backbone for the TJs but also seems to play a role in regulation of paracellular transport. Finally, JAM family proteins are involved in TJ assembly and also regulate the function of the intestinal barrier. Transmembrane proteins are connected to the actin cytoskeleton through the plaque proteins called Zonula occludens-1,2,3 proteins (ZO-1,2,3) or partitioning defective adaptor proteins (PARs). Plaque proteins allow the stabilization and clustering of transmembrane proteins but are also

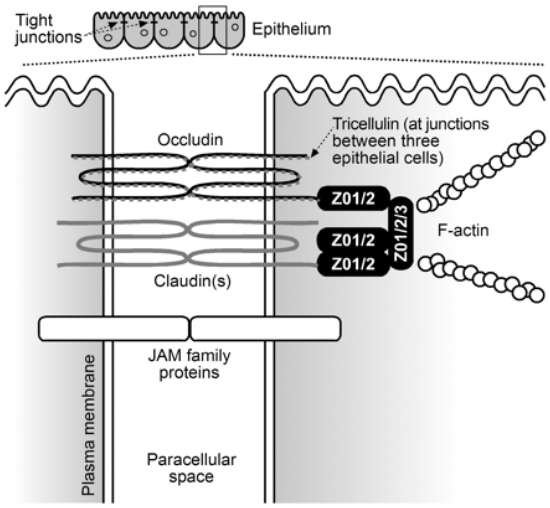

Figure 5: Structure of tight junctions. Tight junctions are composed of transmembrane proteins and plaque proteins. Transmembrane proteins such as occludin, claudins and junctional adhesion molecules (JAMs) seal the paracellular space between epithelial cells. Plaque proteins such as Zonula occludens (ZO) proteins allow to link transmembrane proteins to the actin cytoskeleton (adapted from Ulluwishewa et al., 2011)[13].

essential in TJ regulation. Indeed, TJs can be remodeled by the interaction of external stimuli like commensal and pathogenic bacteria, as well as food antigens. Several signaling proteins are involved in this TJ regulation such as protein kinase C, mitogen-activated protein kinases, myosin light chain kinase and Rho GTPases[9,13,14] (**Figures 4 A and 5**).

The **adherens junctions** (AJs) mainly consist of cadherin-catenin interactions. E-cadherin is the main transmembrane protein that associates to α- and β-catenins or p120 catenin. Catenins make a link between cadherin and actin. AJs are particularly involved in cell-cell adhesion and intracellular signaling[15,16] (**Figure 4 B**).

The **desmosomes** (DMs) are subjacent to AJs. They are composed of desmosomal cadherins: desmogleins and desmocollins. These proteins link plakophilins that bind keratin filaments through desmoplakin proteins. Similarly to AJs, DMs are regulators of cell adhesion and intracellular signaling. In addition, they regulate cellular morphogenesis and differentiation[13,17] (**Figure 4 C**).

2. Gut-associated lymphoid tissues

The mucosal epithelium of the gastrointestinal tract is constantly in contact with the commensal microbiota, as well as numerous pathogens such as bacteria, viruses, protozoan parasites or toxins. The intestinal immune system has two major tasks: 1) It has to remain tolerant against commensal microorganisms, food antigens and self antigens. 2) At the same time, it has to be able to rapidly mount protective responses against enteric pathogens. Protection of this surface is ensured by a combination of mechanical, physicochemical and immunological barriers. Mechanical and physicochemical barriers include mucus, lactoferrin, glycocalyx, defensins, peroxidases, as well as peristalsis

Specific immune responses are produced in specialized structures called GALTs[18,19]. GALTs are divided into inductive and effector sites. Inductive sites are sites where antigens are sampled and comprise cryptopatches (CPs), isolated lymphoid follicles (ILFs), mesenteric lymph nodes (MLNs) and

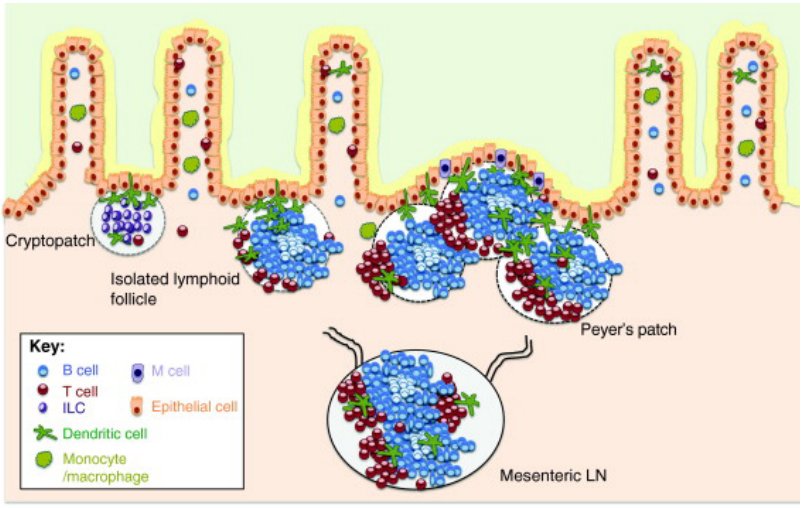

Figure 6: GALT. Cryptopatches are small structures containing innate lymphoid cells and dendritic cells. These can mature in isolated lymphoid follicles containing B cells and germinal centres. Peyer's patches are lymphoid aggregates consisting in organized T and B cell areas (Adapted from Pearson et al., 2012)[20].

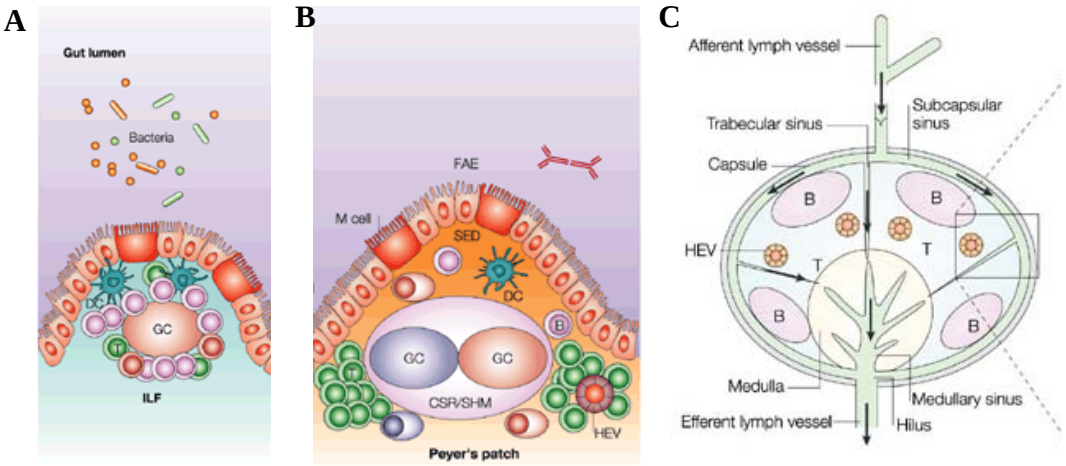

Figure 7: Schematic representation of GALTs. Isolated lymphoid follicles (ILFs), Peyer's patches (PPs) and mesenteric lymph nodes (MLNs) are GALTs with organized lymphoid structures. A. and B. ILFs and PPs are composed of follicle-associated epithelium (FAE) containing microfold cells (M cells) that sample antigens and a subepithelial dome (SED) rich in dendritic cells (DCs) and B cell follicles that contain germinal centres (GCs). Interfollicular areas contain T cells but also high endothelial venule (HEVs) where migration of naive cells takes place. C. MLNs are also composed of B cell zones with GCs, regions containing T cells and HEVs. Antigens are translocated into the MLNs via lymph vessels. Interstitial fluid passes through trabecular and subcapsular sinuses that converge into the medullary sinuses (adapted from Fagarasan and Honjo, 2003; Von Adrian and Mempel, 2003)[21,22].

Peyer's patches (PPs), this latter is the most organized structure (**Figure 6**). Effector sites are where cellular responses mediated by T cells and humoral responses take place. These sites are located within the lamina propria and the intestinal epithelium[23].

2.1 Cryptopatches and isolated lymphoid follicles

CPs and **ILFs** are small structures found in the small intestine and colon with an average of 30 000 CPs and ILFs in humans[20,24].

CPs mainly contain innate lymphoid cells such as lymphoid tissue inducer cells, common lymphoid progenitors ($Lin^{-}cKit^{+}Il\text{-}7R\alpha^{+}$) and dendritic cells (DCs). Lymphoid tissue inducer cells orchestrate the development of GALTs. They play a crucial role in the recruitment of B and T cells leading to the maturation of CPs into ILFs through signaling from commensal bacteria (**Figure 6**).

ILFs are found in the small intestine and colon of mice and humans. They are constituted of a follicle-associated epithelium (FAE) that contains microfold cells (M cells) specialized for antigen sampling and a subepithelial dome (SED) where abundant B cell zones containing germinal centres are found[21] (**Figures 6 and 7 A**).

2.2 Peyer's patches

PPs are aggregated lymphoid follicles. More than 100-300 PPs are irregularly distributed in the small intestine of humans with the greatest density of them found in the ileum and jejenum. In mice, between 5 and 14 PPs are present in the ileum and jejunum[20,25]. PPs are composed of three distinct domains: the FAE, the follicular area and the interfollicular area. The **follicular area** contains B cells, as well as follicular DCs (FDCs) and macrophages. **The interfollicular area** is composed of T cells, interdigitating DCs (IDCs) and macrophages. The **FAE** of PPs differs from the epithelium at other sites. The mucus layer is thinner, the digestive enzymes are lightly expressed and the composition of glycocalyx is particular. The main feature of FAE is the presence of M cells that are enterocytes poorly endowed with brush-border specialized in transcytosis of luminal material[26]. Finally, located between the FAE and the

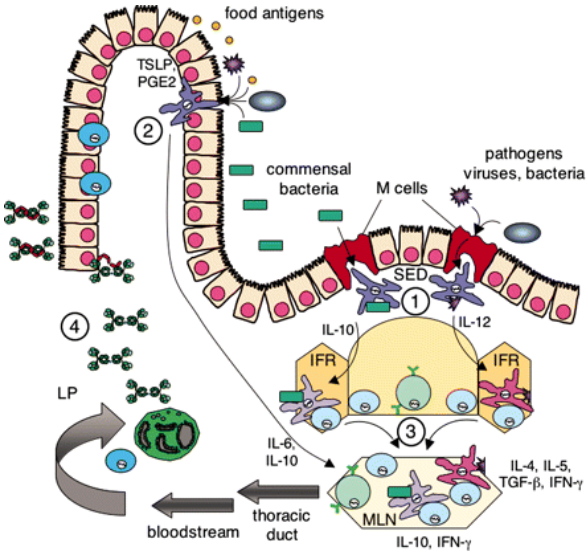

Figure 8: Induction of immune responses in GALT. 1) M cells sample luminal antigens and transfer them to DCs. After their maturation, DC migrate to the T cell zones (interfollicular regions, IFR). 2) Intraepithelial DCs are also able to directly uptake antigens by extending their dendrites through tight junctions and then migrate to the MLNs. 3) Depending on the context (inflammation, tolerance), various cytokines are produced by immune cells. 4) Activated T cells stimulate B cells and, in the lamina propria, plasma cells produce polymeric IgA that are translocated as SIgA (Adapted from Corthésy et al., 2007)[18].

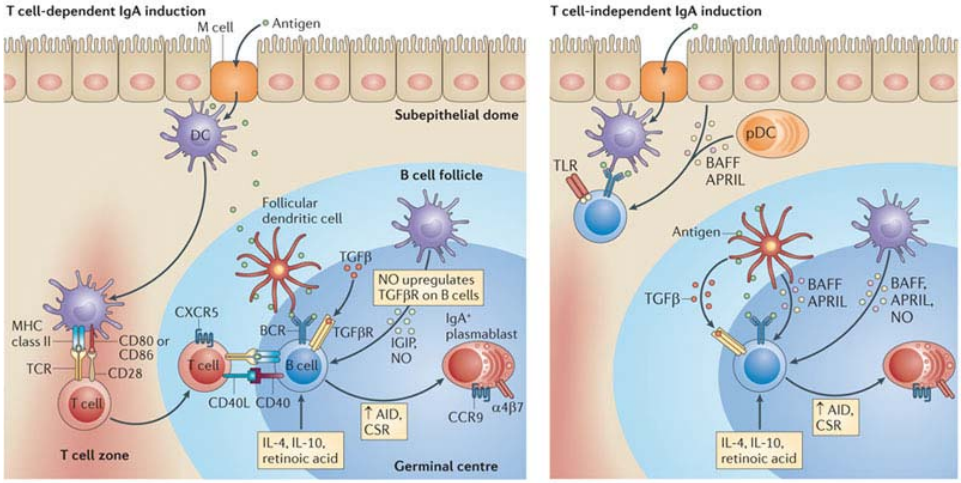

Figure 9: T cell-dependent and - independent IgA-induction in Peyer's patches. Antigens are captured by M cells and sample by DCs in the SED. In T cell-dependent IgA induction, activated DCs stimulate T cells. Effector T cells enter the B cell follicles and activate B cells via receptor mediated-interaction, as well as by secreting IgA-inducing cytokines. Nitric oxide (NO) secreted by DCs upregulated the expression of TGF-β receptor (TFGβR) on B cells. Finally, the expression of activation-induced cytidine deaminase (AID) is induced, then class-switch recombination (CSR) occurs. In T cell-independent IgA response, DCs and FDCs directly activate B cells in SED and in follicles by presenting antigens and by secreting cytokines such as BAFF and APRIL leading to CSR (adapted from Pabst et al., 2012)[29].

follicular area, a SED is present which contains plasma cells, T and B cells, as well as macrophages, monocytes and IDCs[25] (**Figures 6 and 7 B**).

2.3 Mesenteric lymph nodes

MLNs are encapsulated structures present along the GI tract. Their architecture shared with other types of lymph nodes can be divided into two main sites: the cortex and medulla. The **cortex** is splitted into the paracortex (region with diffused T cells) and B cell areas containing primary follicles and germinal centres formed after encounter with an antigen. Free antigens or antigens within DCs are transferred to the lymph node from peripheral tissues via afferent lymphatic vessels and naive lymphocytes enter the lymph node from the blood via high endothelial venules (HEVs). The paracortex is the region where T cells and DCs interact, while humoral responses mainly take place in these B cell zones. An important network of lymph draining sinuses is present in the **medulla**. Interstitial fluid passes through trabecular and subcapsular sinuses that converge into the medullary sinuses. The sinuses are separated by medullary cords containing plasma cells, macrophages and memory T cells[22] (**Figures 6 and 7 C**).

2.4 Induction of immune responses in GALTs

T cell activation

M cells capture antigens in the lumen and convey them to the SED for sampling by DCs or elimination by macrophages. After antigen capture, DCs mature and migrate to T cell zones (interfollicular region, IFR) in PPs, ILFs or MLNs. A subset of DCs called intraepithelial DCs are also able to directly take the antigens in the intestinal lumen by extending their dendrites through tight junctions before migrating to the MLNs. In all cases, DCs activate naive T cells that differentiate into effector cells (**Figures 8 and 9 (left panel)**). Depending on the cytokine environment, various subsets of T cells are generated: T helper (Th) 2 mainly involved in Ab response, Th1 mainly require in intracellular pathogen responses, regulatory T cells that lead to tolerance[27] and Th17 cells that can play pathogenic or protective roles depending on the context[28].

B cell activation and antibody response

1) **T cell-dependent Ig-induction**: activated T cells, mainly Th2 cells, enter the B cell follicles to stimulate B cells via a receptor-mediated interactions and secretion of IgA-inducing cytokines. Subsequently, B cells proliferate into germinal centres and activation-induced cytidine deaminase (AID) is expressed in B cells leading to IgA class-switch recombination[29,30] (**Figures 8 and 9 (left panel)**). In addition, transforming growth factor beta (TGF-β) seems to be particularly important in class switching to IgA. In this context, DCs are able to increase the expression of the TGF-β receptor on B cells by producing nitric oxide (NO) (**Figure 9 (left panel)**).

2) **T cell-independent Ig-induction**: in this case, luminal antigens are directly sampled by M cells, transferred to DCs present in SED or to FDCs, which subsequently present them to B cells. The secretion of cytokines such as B-cell activating factor (BAFF), a proliferating-inducing ligand (APRIL) and TGF-β by DCs leads to IgA class-switch recombination (**Figure 9 (right panel)**).

Finally, IgA^+ B cells migrate through the bloodstream to effector sites and in the lamina propria, IgA^+ B cells differentiate into plasma cells that produce and secrete dimeric IgA and pIgA[29,30]. Dimeric IgA (dIgA) and pIgA are then transported across the epithelium by the polymeric immunoglobulin receptor (pIgR) expressed on the basolateral surface of mucosal epithelial cells. At luminal surfaces, secretory IgA (SIgA) is released as a complex of pIgA and the cleaved extracellular domain of the pIgR, called secretory component (SC)[31,32] (**Figure 8**). Even though SIgA is the most abundant Ab found at mucosal surfaces[33,34], plasma cells also produce pentameric IgM that will be secreted in the form of secretory IgM (SIgM) in the same way as SIgA[35].

3. Immunoglobulin A

Among all immunoglobulin (Ig) subclasses, IgA is the most abundantly produced Ig (66 mg/kg/day in humans)[33]. It is the main antibody found at mucosal surfaces in the form of SIgA and is the second most prevalent in serum, due to IgA production by bone marrow plasma cells[36].

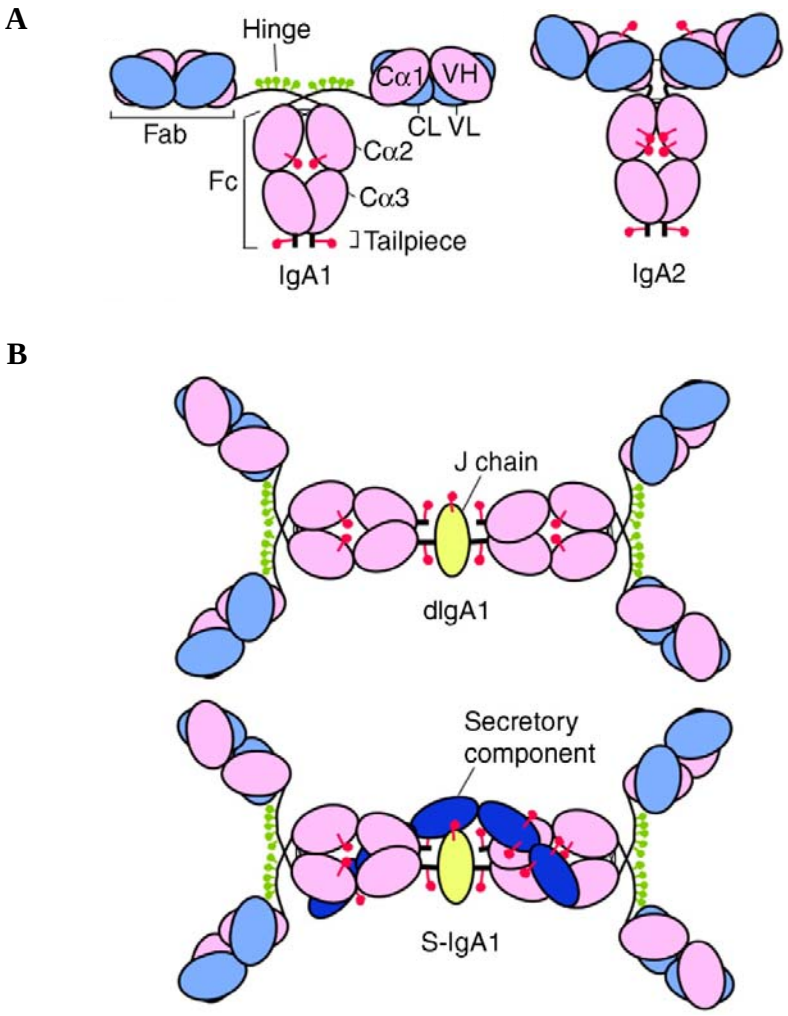

Figure 10: Structure of human IgA. A. IgA1 and IgA2. B. Dimeric IgA1 (dIgA1) and SIgA1. IgA heavy-chain domains are shown in pink, light-chain domains in light blue, J chain in yellow and secretory component in dark blue. N- and O-linked oligosaccharides are shown in red and green, respectively (adapted from Woof and Russell, 2011)[31].

3.1 Structure

IgA can be found in three different molecular forms: monomeric, dimeric or polymeric. The general arrangement of monomer is similar to all Abs composed of two identical Fab regions and a Fc region. The monomer is formed of two heavy alpha chains composed of four globular domains (VH, Cα1, Cα2, Cα3) and two light chains (kappa or lambda chains) composed of two globular domains (VL and CL). The structure is stabilized by inter-chain disulphide bonds[31]. In addition, IgA is characterized by an elongation of the α chain C-terminus composed of 18 amino acids called tailpiece[37] (**Figure 10 A**).

In humans and higher apes, two subclasses have been described: IgA1 and IgA2. In addition, IgA2 is observed in two allotypic forms, IgA2m (1) and IgA2m(2). A third form IgA2m(3) was also described but has been less studied[38]. The various subclasses and allotypic versions differ by the arrangement of their disulphide bridges. Disulfide bonds are present between the heavy and light chains in IgA1 and IgA2m(2), whereas these disulfide bridges are absent between the heavy chains but exist between the light chains in IgA2m(1). IgA1 is characterized by a 16-amino acid insertion forming a hinge that carries between 3-6 O-linked oligosaccharides. These oligosaccharides are mainly constituted of sialic acid, galactose and *N*-acetyl galactosamine. Both IgA1 and IgA2 heavy-chain domains carry N-linked oligosaccharides. IgA1 carries these glycans on the Cα2 domain (Asparagin (Asn) 263) and the tailpiece (Asn 459). IgA2m(1) has additional glycans bound to Asn 166 of the Cα1 domain and Asn 337 of the Cα2 domain (**Figure 10 A**). Finally, IgA2m(2) bears supplementary glycans at Asn 211 of Cα1 domain. Glycans provide 6-10% of molecular mass of IgA[31,37,39,40,].

In dIgA, a single joining (J) chain of 15-16 kilodalton (kDa) binds two monomers via tailpiece. The J chain has eight Cystein (Cys) residues. Six residues form intra-chain disulfide bonds, whereas Cys 14 and Cys 68 of J chain binds the tailpieces (Cys 471) of each monomer. This polypeptide is also decorated by a N-linked oligosaccharide at Asn 48. In addition, the J chain directly interacts with pIgR and is required for the association of pIgA with SC to generate SIgA[31,37,41] (**Figure 10 B**).

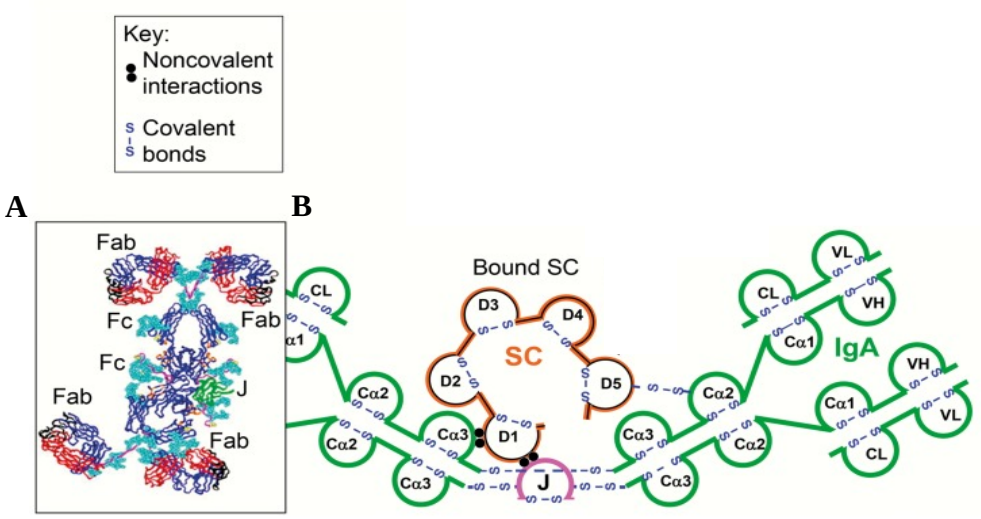

Figure 11: Tertiary structure of dIgA and secondary structure of SIgA showing covalent/non-covalent interactions in SIgA. A. The tertiary structure of dIgA shows the Fab and Fc regions, as well as the J chain. B. Non-covalent interactions (black circles) are present between the J chain and the domain D1 of SC, as well as between the domain D1 of SC and the domain Cα3 of one IgA subunit. Disulphide covalent bonds (s-s) exist between Cys 467 or 502 in the domain D5 of SC and Cys 311 in the Cα2 domain of one IgA subunit (adapted from Brandtzaeg, 2013)[42].

In SIgA, SC derived from pIgR is a glycoprotein of 70-80 kDa with five Ig-like domains (D1-D5)[37]. Seven surface N-oligosaccharides are exposed and represent 20% of its molecular weight[43]. SC is bound to pIgA through non-covalent and covalent interactions. Non-covalent bonds take place between the domain D1 of SC and the J chain and between the domain D1 of SC and the Cα3 domain of one IgA subunit. Covalent binding is present between the domain D5 of SC (Cys 467 or 502) and the domain Cα2 of the other IgA subunit (Cys 311)[42,44]. The domains D2 and D3 of SC are involved in the formation of this covalent bond by properly positioning the domain D5[45] (**Figure 11**).

Distribution of IgA molecular forms and subclasses

The abundance of molecular forms and IgA subclasses varies according to the sites in the body. While IgA is mainly released in dimeric or polymeric form in mucosal secretions, it is mainly found as monomers in serum (85-90% of total serum IgA). At mucosal sites, the subclass proportion varies: 80-90% IgA1 in nasal and male genital secretions; ca. 60% IgA1 in saliva; to 60% IgA2 in colostrum and female genital secretions. In serum, the subclass IgA1 is predominant (ca. 90% IgA1)[31,36].

3.2 Roles of SIgA at mucosal surfaces during infections

SIgA is a multi-facet Ab with numerous roles in defense against infections and in homeostasis of mucosal surfaces. Indeed, it is essential in the control of host-commensal relationship and its interaction with M, epithelial cells and DCs allows a modulation of inflammatory responses[46]. The next pages are deliberately focused on the role of SIgA in protection against bacterial and toxin infections because the thesis work mainly deals with these aspects.

The main mechanism of protection conferred by SIgA is the neutralization of pathogenic microorganisms and antigens by a process called immune exclusion and their clearance by peristalsis. Immune exclusion allows to prevent contacts between the pathogenic microorganisms and mucosal surfaces, as well as a subsequent invasion of mucosal tissues. Studies using *in vitro* and *in vivo* models strongly demonstrated the primordial role of this mechanism against bacterial and toxin infections[19,46].

The use of *in vitro* reconstituted polarized epithelial cell monolayers that mimick mucosal epithelium has been a crucial tool to dissect the ability of immune exclusion of IgA/SIgA[46]. Specific anti-cholera toxin IgA efficiently blocked the binding of the toxin to intestinal epithelial cell monolayers and subsequently, prevented the toxin-induced response of enterocytes[47]. Monoclonal IgA directed against ricin allowed neutralization of ricin and protection of epithelial cells monolayers[48]. In the lab, similar results were obtained with IgA specific for *Clostridium difficile* (*C. difficile*) toxin A[49]. Interestingly, in this study, the various molecular forms of IgA were tested and it is interesting to underline that pIgA was superior to monomers to neutralize toxin A and consequently, to protect epithelial cell monolayers. The neutralization of bacteria by IgA was also assessed. Monoclonal anti-*Salmonella* IgA prevented binding of bacteria and subsequent infection of epithelial cell monolayers[50]. Finally, in order to examine the possibility to use polyclonal SIgA against infections, Cravioto et al. (1991)[51] and Carbonare et al. (2005)[52] showed that human colostrum- and milk-derived SIgA were able to inhibit the adhesion of enteropathogenic *Escherichia coli* (*E. coli*) to epithelial cell monolayers and to prevent cell invasion.

The immune exclusion properties of SIgA were also demonstrated using *in vivo* models. Passive oral administration of specific anti-lipopolysaccharide (LPS) IgA directed against *Vibrio cholerae* (*V. cholerae*) prevented *V. cholerae*-induced diarrhea in mice[53]. Specific monoclonal IgA preincubated with *Helicobacter felis* neutralized the bacteria, which in turn did not colonize mice[54]. Similar results were observed with *Shigella flexneri* (*S. flexneri*) and *Salmonella typhimurium*. Specific anti-LPS IgA against *S. flexneri* protected from experimental shigellosis in mice[55] and specific anti-LPS IgA prevented from oral[56] or intranasal *Salmonella* challenge[57]. Interestingly, passive administration of polyclonal SIgA protected mice against infections. Indeed, intranasal delivery of human colostrum-purified SIgA led to protection of mice against pulmonary *Mycobacterium tuberculosis* infection[58]. In the context of luminal pathogen-neutralization, the presence of SC is essential to SIgA to reduce its sensitivity to proteases and acid[59,60]. Moreover, SC ensures a correct localization of the molecule within the lumen[61]. The presence

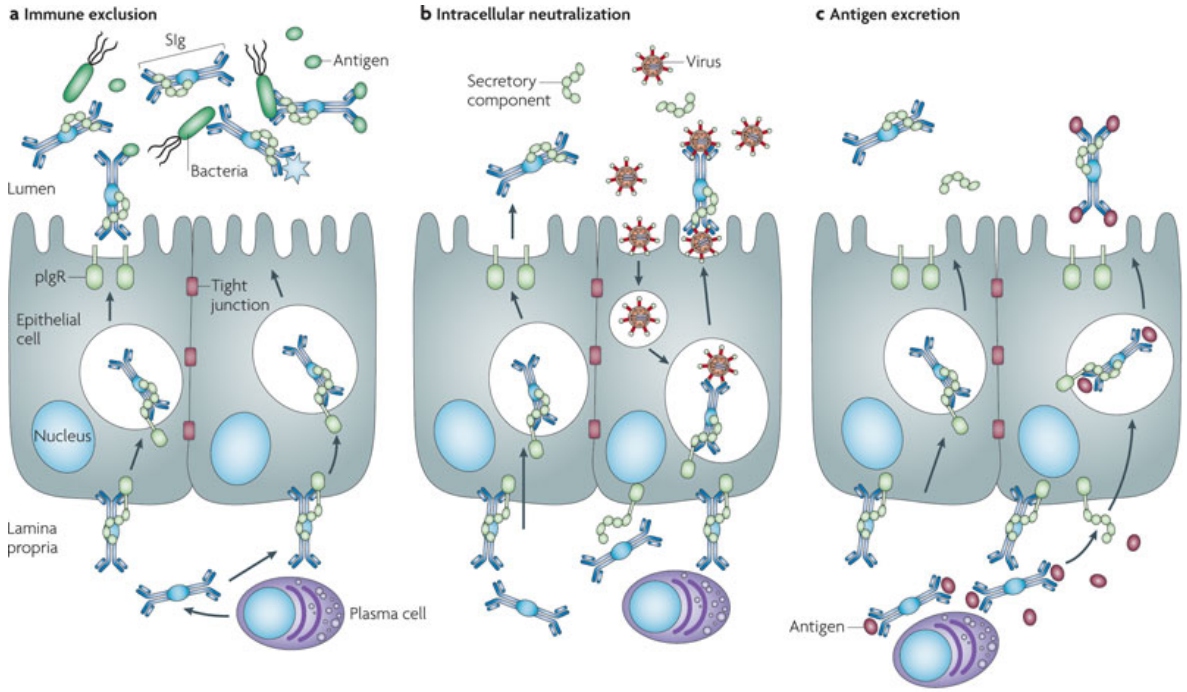

Figure 12: Roles of SIgA in protection of mucosal surfaces. A. SIgA mainly acts by immune exclusion in the lumen and allows the antigen clearance by peristalsis. B. SIgA is able to neutralize intracellular antigens like viruses. C. SIgA is able to bind an antigen in the lamina propria, transports it through epithelial cells and excretes it to the luminal side using transcytosis by pIgR (Adapted from Strugnell and Wijburg, 2010)[62].

of N-glycans is thought to be crucial for the interaction with the mucus [46] and plays a role in interaction with toxins and bacterial compounds such as adhesin[63].

In addition to the role of SIgA in neutralization of pathogenic antigens in the lumen, pIgA is also able to bind antigens in the lamina propria, transports them through epithelial cells and excretes them to the luminal side using transcytosis by pIgR[64]. Moreover, SIgA is involved in intracellular neutralization of infectious agents such as viruses[65] and of proinflammatory antigens in the apical recycling endosome compartment[66] (**Figure 12**). Finally, SIgA has anti-inflammatory properties. A study highlighted this phenomenon using a rabbit ileal loop model for *S. flexneri* infection. The authors demonstrated that bacteria were neutralized by specific anti-LPS SIgA but some immune complexes entered the PPs and were internalized by PP DCs. Remarkably, immune complexes composed of SIgA down-regulated inflammatory responses and prevented damage of the intestinal barrier[67].

3.3 Description of enteric infections and the roles of IgA/SIgA in the control of specific infections

Shigella flexneri

Shigella is a gram-negative facultative anaerobe belonging to *Enterobacteriacceae* family. This genus is divided into four species: *S. flexneri, S. sonnei, S. boydii* and *S. dysenteriae*. *S. flexneri* is the major ethiological agent of shigellosis and is divided into 13 serotypes according to the O-antigen side chain of outer membrane LPS. Shigellosis is a diarrheal disease causing over 160 million cases per year and 1.1 million deaths, mainly in developing countries[68].

Infections are transmitted by fecal-oral route via ingestion of contaminated food and only 10-100 bacteria are sufficient to cause disease[69]. Once in the gastrointestinal tract, bacteria mainly translocate across the epithelium by transcytosis through M cells[70]. However, a translocation independent of M cells by paracellular passage through the intestinal barrier was also demonstrated. In this process, the bacteria modulate the function of tight junctions[71]. Once in PPs, they invade macrophages, multiply within their

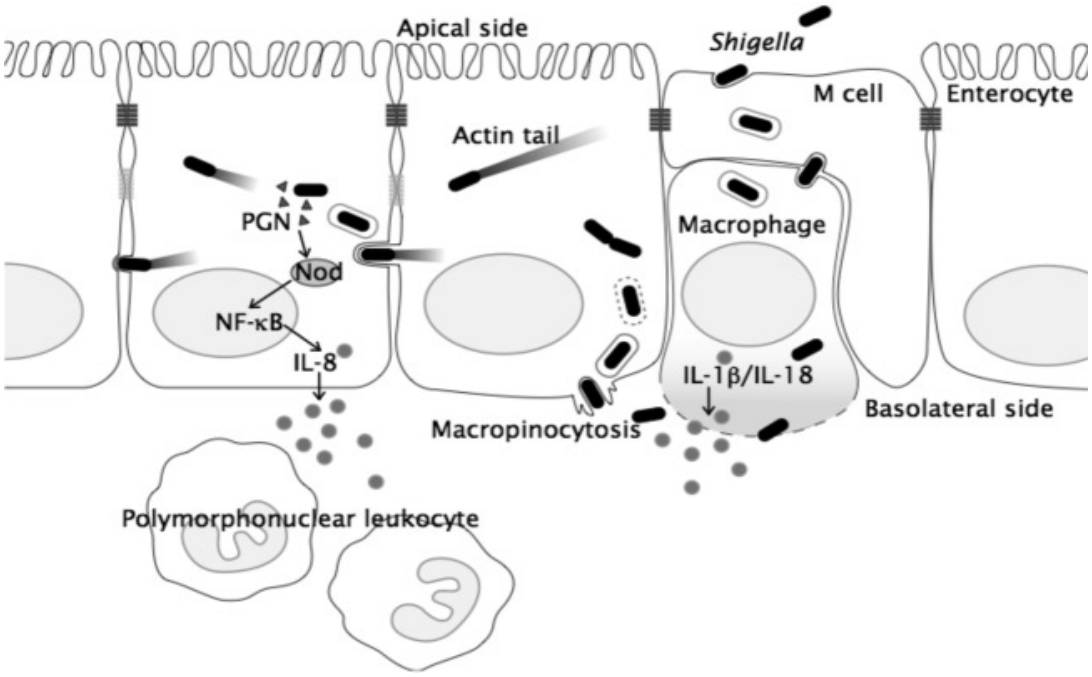

Figure 13: *Shigella* infection of intestinal epithelium. *Shigella* translocates across the epithelium by transcytosis through M cells and invades macrophages. It multiplies within their cytoplasm, which results in massive inflammation (release of IL-1β/IL-18) and finally induction of macrophage's death. The bacteria released from killed macrophages are then able to invade the contiguous intestinal cells from the basolateral surface of the epithelium. Within epithelial cells, they disrupt the vacuole membrane surrounding them, disseminate into the cytoplasm of cells and spread into the adjacent epithelial cells with cytokine release. Spreading cell by cell, bacteria release LPS, peptidoglycan (PGN), or other virulent factors recognized by pattern-recognition receptor like Nucleotide-binding oligomerization domain (NOD) leading to secretion of IL-8 trough NF-κB activation (Adapted from Sasakawa, 2010)[72].

cytoplasm, resulting in initial massive inflammation (e.g. IL-1β, IL-18 release), followed by induction of apoptosis. The bacteria released from killed macrophages are then able to invade the contiguous enterocytes from the basolateral side of the epithelium. Within epithelial cells, they disrupt the vacuole membrane that surround them, disseminate into the cytoplasm of cells and spread into the adjacent epithelial cells[69,72,73,74]. Epithelial cells respond by producing chemokines (mostly IL-8) that recruit polymorphonuclear cells involved in clearance of bacteria[75] (**Figure 13**).

The molecular "equipement" crucial for bacterial invasion and survival is encoded by a virulence plasmid of 200 kilobase (kb). A conserved plasmid region of 31 kb was demonstrated to be sufficient to invade intestinal epithelium and kill magrophages. This region is composed of 32 genes organized into two loci: the *ipa* locus and *mxi-spa* locus. The first locus encodes for secreted proteins called "invasion plasmid antigens" (Ipa): IpaA, IpaB, IpaC, IpaD[68,69,76]. These key virulence factors are secreted upon contact of bacteria with epithelial cells and mainly allow the entry into host cells. In addition, IpaB, C and D were shown to induce actin polymerization at the site of attachment[77] and lysis of the vacuoles[78]. The second operon encodes for the constituents of a type-III secretion system (T3SS): membrane expression of Ipa (mxi) and surface presentation of Ipa (spa) antigens[68,69]. T3SS is a needle-like structure allowing the translocation of more than 50 effector proteins[72] such as Ipa proteins from the bacterial cytoplasm into the host cell. Finally, the virulence plasmid also encodes for two transcriptional activators, VirB and MxiE, that control the transcription of T3SS-associated genes and four chaperones (IpgA, IpgC, IpgE, Spa15) that also have a regulatory role[68,69]. The contact with the intestinal epithelium leads to the activation of T3SS and effector proteins are secreted into host cells. These effectors stimulate signaling pathways involved in actin polymerization leading to the formation of large membrane ruffles that favor the endocytosis of bacteria into the host cell. Once endocytosed, bacteria move from one cell to another one thanks to a machinery allowing polymerization/depolymerization of actin. Indeed, bacteria are devoid of a flagellum and assemble an outer membrane protein called VirG, as well as N-WASP, Arp2/3

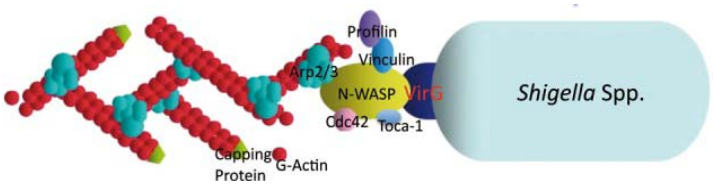

Figure 14: *Shigella* motility dependent on actin. The machinery necessary for *Shigella* motility is composed of VirG, N-WASP, Arp2/2 complex, Profilin and Toca-1. This machinery is formed at one pole of the bacterium to act on actin polymerization (adapted from Sasakawa, 2010)[72].

complex, Profilin and Toca-1 at one pole[72] (**Figure 14**). In addition, bacteria recruit the ZO-1 tight junction protein to form their actin tail[79]. Finally, once entered the host epithelial cells, bacteria circumvent the host immune response by modulating inflammatory responses, escaping from autophagy and extending the intestinal epithelial cell life[72].

Studies in animal models and with samples from infected humans have suggested that a serotype-specific humoral immune response is the main component involved in protection against shigellosis with systemic and mucosal responses against LPS and proteins encoded by the virulence plasmid[68]. Consistent with the predominance of SIgA at mucosal surfaces, it was shown that the number of anti-LPS IgA secreting cells increased in infected patients[81]. Clemens et al., (1986)[82] showed a decrease of severity of shigellosis in infected infants who received breast milk of mothers previously exposed to the pathogen. Anti-LPS SIgA seems to be involved in this partial protection. Studies using animal models demonstrated that a monoclonal dIgA specific for *S. flexneri* serotype 5a LPS called SIgAC5 led to the protection from *S. flexneri* infection. Indeed, SIgAC5 was sufficient to protect animals against a mucosal challenge by two main mechanisms: immune exclusion[55,61] and decrease of inflammatory responses[67]. In addition, SIgAC5 was shown to directly act on the bacterial virulence by suppressing the activity of the T3SS. Consequently, it reduced the secretion of IpaB and IpaC, as well as the bacterial membrane potential and intracellular adenosine triphosphate levels[83].

Clostridium difficile infection (focus on toxin A)

C. difficile is a gram-positive bacterium that belongs to the *Clostridium* genus comprising 181 species. Clostridia are present in the environment and in the gastrointestinal tract of humans and of many animals. Several species can be pathogenic such as *C. difficile* that is the primary cause of nosocomial antibiotic-associated diarrhea and colitis[84,85,86].

C. difficile enters the organism via the fecal-oral route either in vegetative form or in spore form that survive in the acidic environment of the stomach. In the small intestine, spores develop into vegetative

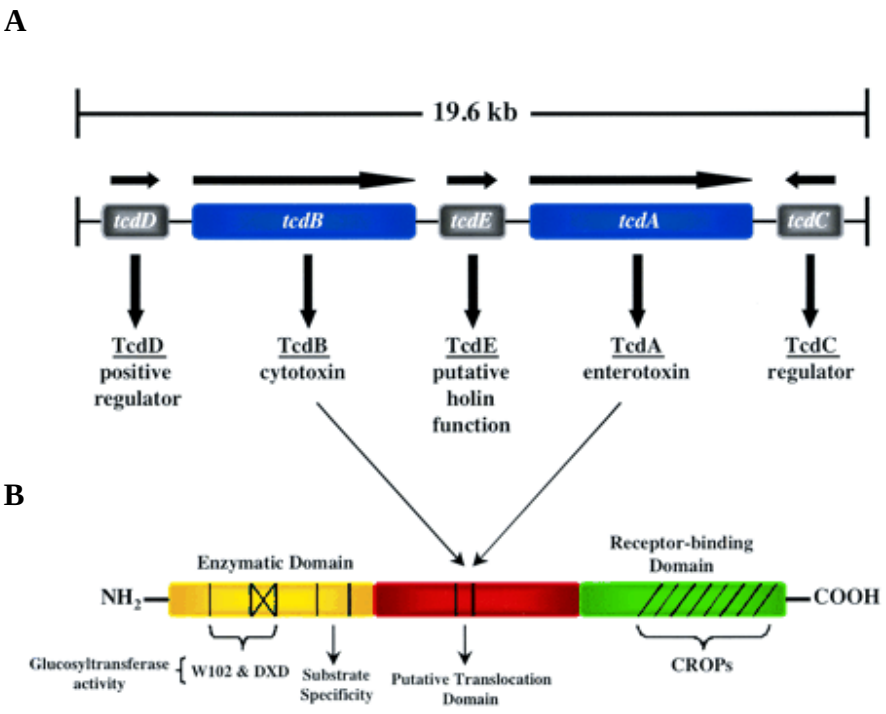

Figure 15: *Clostridium difficile* pathogenicity locus and protein domain structures of TcdA and TcdB. A. TcdA and TcdB are encoded on the 19.6 kb pathogenicity locus. Three additional open reading frames are present: tcdD, a putative positive regulator; tcdC, a putative negative regulator and tcdE, a proposed holin protein. B. Toxins are composed of three domains: the C-terminus domain is involved in receptor-binding. The N-terminus domain is the enzymatic domain including the glycosyltransferase activity. The middle domain is a putative translocation domain involved in membrane transolocation (Voth and Ballard, 2005)[80].

form and *C. difficile* proliferate in the large intestine of patients with a disrupted microbiota[87]. Its pathogenicity is mainly based on the secretion of toxins A (308 kDa) and B (269 kDa). Toxin A and B are encoded by *tcdA* and *tcdB* located in a 19.6 kb pathogenicity locus, the PaLoc composed of five genes: *tcdA*, *tcdB*, *tcdC* - encoding for a potential negative regulator - *tcdD* - encoding for a potential positive regulator - and a holing-like pore-forming protein, *tcdE* (**Figure 15 A**). Both toxins are glucosyltransfereases and are composed of three parts: N-terminal, C-terminal and central regions. The N-terminal catalytic domain provides the biological activity to toxins. The C-terminal domain is involved in receptor binding (**Figure 15 B**). The receptor is not known but the presence of carbohydrates in toxin A seems to be important for binding. Finally, the hydrophobic central domain seems to be involved in membrane translocation process[80,88,89]. Uptake of toxins begins with the binding to the potential receptor via the C-terminal domain; then, the acidification of endosome induces a refolding of the toxin leading to an increase in hydrophobicity. The presentation of hydrophobic regions allows membrane penetration and formation of pores. Finally, the N-terminal catalytic domain is released from the endosome and enters the cytosol of intestinal epithelial cells[89]. Once entered, these toxins inactivate small GTPases (Rho, Rac and Cdc42) by monoglycosylating GTPases with uridine diphosphate (UDP)-glucose as co-substrate[80,90]. Rho, Rac and Cdc42 regulate several crucial cellular processes such as actin polymerization and maintenance of tight junctions ensuring cellular structural integrity but also cell cycle and signalization via mitogen-activated protein kinases. Thus, the main consequences of toxin activity are actin condensation, alteration of structural integrity and finally, cell apoptosis[80] (**Figure 16 A**). In addition, toxin A leads to neutrophil infiltration, release of inflammatory cyto- and chemokines such as IL-8[91], growth regulated oncogen (GRO)-alpha and monocyte chemoattractant protein (MCP)-1[92], as well as production of reactive-oxygen species. Both toxins induce disruption of tight junctions[80,93], as highlighted by a loss of ZO-1, ZO-2 and occludin[94] (**Figure 16 B**). Toxin A is an enterotoxin mainly causing fluid secretion and hemorrhage in animal models, whereas toxin B is a cytotoxin showing cythopathic effects in cell culture but showing little enterotoxicity in animals. It is generally accepted that toxin B access to enterocytes through the effect of toxin A[49].

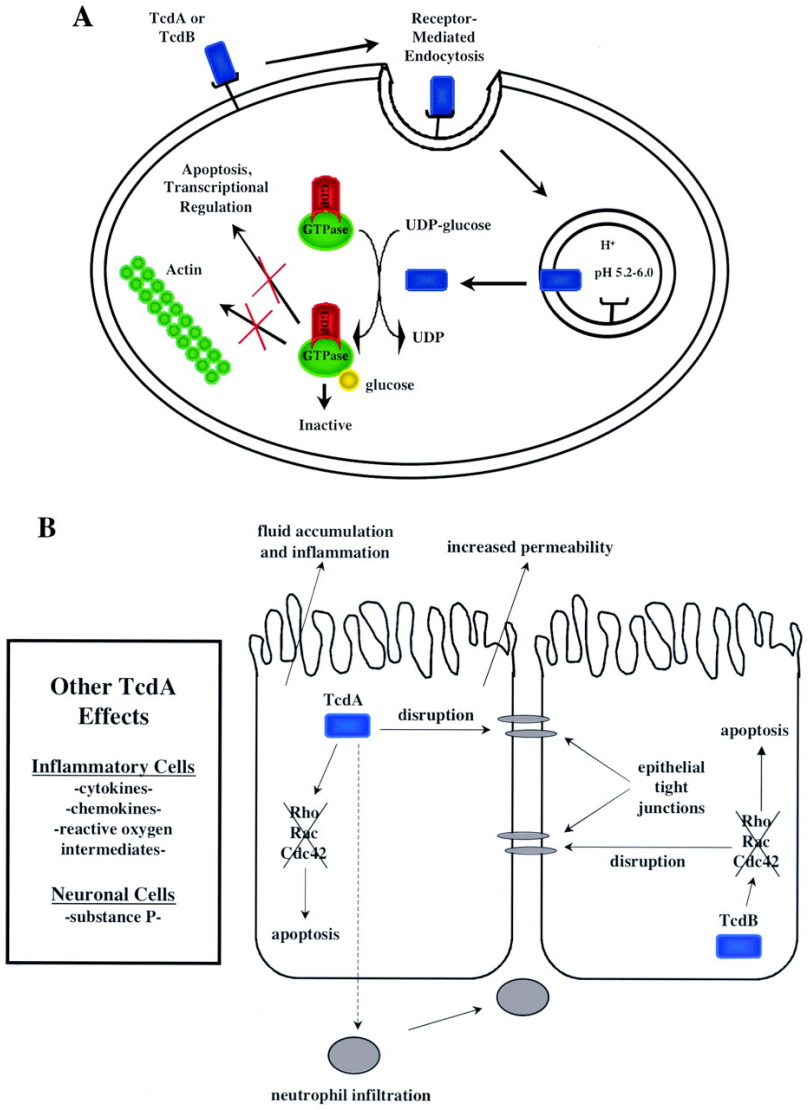

Figure 16: Effects of toxins A and B. A. Intracellular effects induced by toxin A (TcdA) and toxin B (TcdB): after receptor-mediated endocytosis, the toxins act as glycosyltransferases. They inactivate Rho GTPases via a sugar moiety transfer, which leads to actin condensation and apoptosis of the cell. B. Downstream effects of toxin A and B in intestinal epithelial cells: toxin A leads to neutrophil infiltration, as well as chemokine release and reactive-oxygen species production. Both toxins lead to disruption of tight junctions. The consequence is a fluid accumulation and inflammation in the host (Adapted from Voth and Ballard, 2005)[80].

The level of Ab responses to toxins, and mainly toxin A, was shown to be related to the severity and the duration of clinical manifestations. Indeed, acutely ill patients show higher anti-toxin A titers in sera compared to healthy patients[95] and the inability of a patient to mount an Ab response to the toxins can be essential for the predisposition to recurrent episodes of disease after a primary resolution of symptoms[96]. Moreover, studies performed in hamster model showed that passive immunization with anti-toxin A Abs[97] or with avian anti-toxin A and B Abs[98] led to protection. Regarding the role of IgA, it was demonstrated that serum IgA, but not IgG, from patients was able to *in vitro* neutralize toxin A[99]. A few years later, another study carried out with polarized human intestinal cell monolayers, showed that pIgA was superior to mIgA or IgG, even though both specific for toxin A, in prevention of damages caused by toxin A[49]. Finally, in clinical trials, some positive effects such as resolution of diarrhea or relapse prevention were obtained with administration of anti-toxin A and B Abs[100], as well as with intravenous immunoglobulin (IVIg) administration[97].

3.4 Roles of serum IgA

Serum IgA mainly found in the monomeric form seems to have predominantly an anti-inflammatory role[36]. A 30-year-old study demonstrated that human serum IgA inhibited the IgG-dependent phagocytosis of *Candida albicans* blastopores by human polymorphonuclear leucocytes[101]. A more recent study described the same process using human neutrophils, as well as an inhibition of the production of superoxide when antigens are complexed to serum IgA[102]. Human serum IgA was also shown to downregulate the release of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and IL-6 by activated human monocytes[103]. Additional evidences of the anti-inflammatory roles of serum IgA are also illustrated by patients with selective IgA-deficiency who display an increased susceptibility to autoimmune and allergic disorders[36].

FcαRI (CD89)

The discovery of the IgA Fc receptor type 1 FcαRI (CD89) in humans allowed to better understand the anti-inflammatory action of serum IgA[36]. FcαRI is a receptor expressed on monocytes/macrophages,

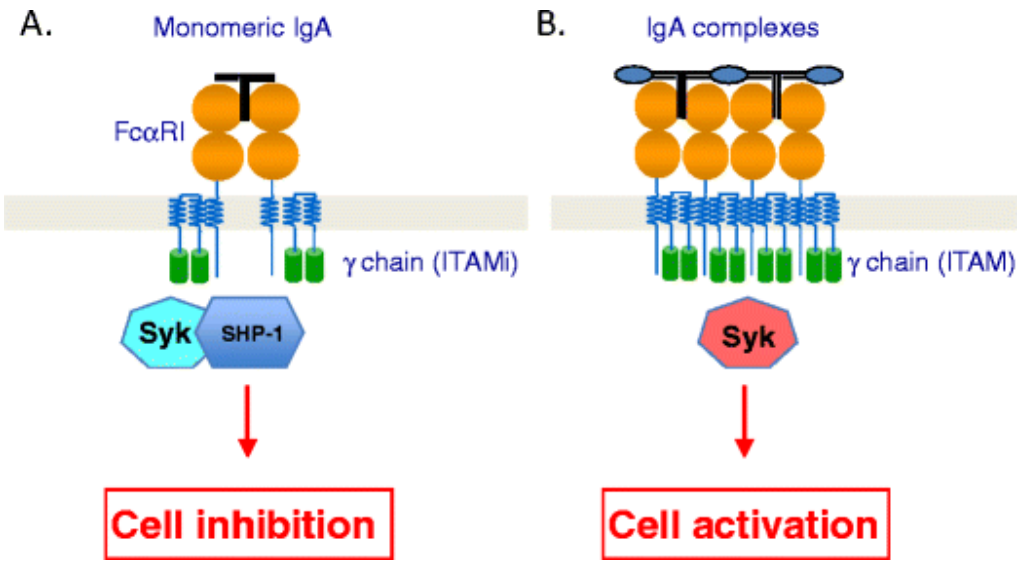

Figure 17: Roles of FcαRI in the immune system. A. Under physiological conditions, mIgA binds to FcRγ-associated FcαRI and induces partial phosphorylation of the FcRγ. This leads to an inhibitory immunoreceptor tyrosine-based activation motif (ITAMi) configuration that allows the recruitment of spleen tyrosine kinase (Syk), then Src homology region 2 domain-containing phosphatase-1 **(**SHP-1). This process conducts to a cellular inhibition state. B. Under pathological conditions, IgA-immune complexes induce FcαRI aggregation and leads to an activating state via an activated ITAM configuration (adapted from Monteiro, 2010)[36].

neutrophils, DCs, liver macrophages (Kupffer cells) and eosinophils[33,36]. FcαRI has a size varying between 55 and 100 kDa according to its glycosylation pattern and is formed by two extracellular Ig-like domains. It is also composed of a transmembrane region and a small cytoplasmic tail. This receptor associates with the signaling FcRγ chain subunit that forms a heterotrimer FcαRI/γγ. Both Fc regions of IgA1 and IgA2 bind to FcαRI. Monomeric IgA poorly binds to FcαRI, whereas pIgA and IgA-immune complexes strongly bind to FcαRI[33]. This receptor plays a dual role in the immune system. Under physiological conditions, serum mIgA binds to FcRγ-associated FcαRI via its domains Cα2 and Cα3[104,105] and allows to transmit inhibitory signals via phosphorylation of FcRγ (**Figure 17 A**). Under pathological conditions, FcαRI is aggregated by IgA-immune complexes that induce cell activation (phagocytosis, superoxide release, cytokine release, antibody-dependent cellular cytotoxicity) through crosslinking of transmembrane FcRγ-associated FcαRI and recruitment of various effectors (**Figure 17 B**). The immunoreceptor tyrosine-based activation motif (ITAM) found on FcRγ adaptor is involved in both above-mentioned processes and mediates the recruitment of Spleen tyrosine kinase (Syk) and Src homology region 2 domain-containing phosphatase-1 **(**SHP-1) leading to inhibitory or activation processes[36]. Interestingly, two types of soluble FcαRI were described. The first type of soluble receptor is associated with pIgA and is found in serum of healthy humans[106]. The second type was found in patients with IgA nephropathy[107].

3.5 IgA receptors

In addition to FcαRI, several other IgA receptors have been identified.

Polymeric immunoglobulin receptor (pIgR)

Polymeric immunoglobulin receptor mainly produced by intestinal epithelial cells (IECs) allows the transcytosis of p/dIgA from the lamina propria into the intestinal lumen. The pIgR is a type I transmembrane protein of approximately 120 kDa and has five homologous extracellular transmembrane domains (domains 1-5), as well as a non-homologous domain (domain 6) and a transmembrane segment. The domain 1 seems to be the most important for IgA binding. This domain is highly conserved, whereas

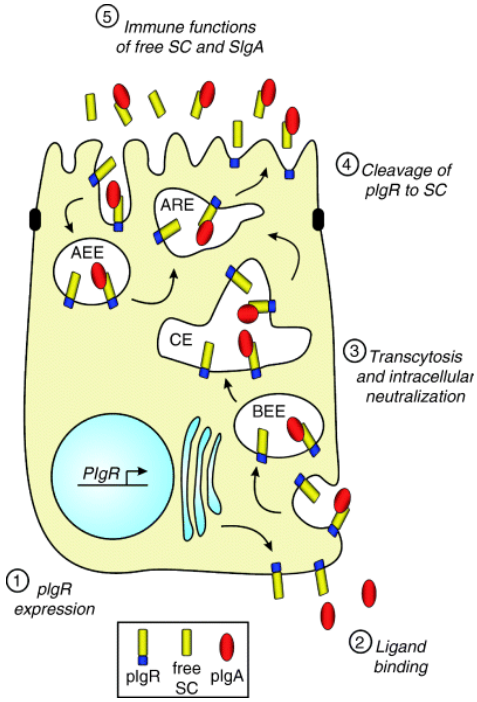

Figure 18: Pathway of pIgR through a polarized epithelial cell. Newly synthesized pIgR is found at the basolateral surface. Polymeric IgA-bound or free pIgR is endocytosed and transported through several intracellular vesicles. At the apical surface, pIgR is cleaved and SC is released. If pIgR transports pIgA, SC remains bound to this one and if pIgR is free, free SC is delivered into the lumen (adapted from Kaetzel, 2005)[108].

the domain 6 is highly variable among species and contains a potential proteolytic cleavages site[109]. The receptor binds dIgA at the basolateral surface of IECs, then receptor-mediated endocytosis of the dIgA-pIgR complex occurs and the transport across the cells is performed via intracellular vesicles. When the dIgA-pIgR complex reaches the apical surface of the cells, the extracellular portion of pIgR is cleaved to give SC, which allows the generation of SIgA. Interestingly, the receptor can be transcytosed in IECs without ligand and the result of cleavage is the release of free SC[108] (**Figure 18**). Several studies described that pIgR expression could be regulated by several cytokines such as interferon (IFN)-γ, TNF, IL-4 and IL-1[110] but also hormones, microbial factors and dietary factors[108].

Fcα/μ receptor (Fcα/μR)

Fcα/μR is a type I transmembrane protein that binds IgA and IgM and was first characterized in mice. Mouse Fcα/μR is constitutively expressed on B cells and macrophages and was firstly described mediating endocytosis of immune complexes with IgM. The human homolog was isolated from a human lymph node complementary DNA library and has 49% of homology with the murine receptor [111]. Human Fcα/μR is expressed on follicular DCs in tonsil, macrophages, plasma cells, intestinal Paneth cells and germinal centres[112].

Fcα/μR and pIgR display some similarities. In particular, the N-terminal Ig-like domain of this receptor shares homology with D1 of pIgR[111]. Mouse Fcα/μR binds mIgA and pIgA, whereas human Fcα/μR binds only the polymeric forms and does neither bind to mIgA, IgG nor SIgA[113]. The Cα3 domain of IgA seems to be essential for the interaction with the receptor[113] .

Transferrin receptor (CD71)

The transferrin receptor (Tfr) is a homodimeric glycoprotein of 760 kDa mediating the uptake of transferrin-iron complexes. Tfr is mostly expressed on hematopoietic cells in fetal liver and bone marrow, but also in lymphocytic and myeloid cells lines[114]. A few years ago, it was found that this receptor was also able to selectively bind IgA1 and that it was upregulated on cultured renal mesangial cells in patients

with IgA nephropathy. This overexpression of Tfr might be involved in IgA deposits in the kidney of these patients[115]. Tfr is also expressed on epithelial cells and, in celiac disease patients, an overexpression and a reorganisation of Trf is involved in retrotransport of SIgA complexes from the apical surface to the lamina propria causing inflammation[116,117,118].

Asialoglycoprotein receptor (ASGP-R)

The ASGP-R is expressed on hepatocyte membrane and participates in the hepatic metabolism of serum glycoproteins including IgA[119]. This receptor seems to be involved in the clearance of IgA and, mainly IgA2, from the blood. This fast clearance of IgA2, and not IgA1, may explain the higher level of IgA1 in serum[120].

M cell IgA receptor

A few years ago, a study demonstrated that human serum or colostrum-derived IgA/SIgA bound mouse M cells and that some M cells of the human ileum were coated with IgA. As ASPG-R or other lectin-like receptors were not expressed on the apical surface of M cells, the presence of (an) IgA-specific receptor(s) on the apical surface of M cells was suggested[121]. Recently, Rochereau et al. (2013)[122] identified that Dectin-1 receptor was expressed on M cells and was involved in SIgA transcytosis.

Secretory component receptor (SCR)

A functional receptor specific for SC expressed on the eosinophil membrane has been described. SC and SIgA interact with this receptor, which induce degranulation of eosinophils, whereas serum IgA does not induce this phenomenon[123]. SIgA also induces degranulation of IL-3 primed basophils that might express SCR[124].

Fc receptor like 4 (FcRL4)

FcRL4 belongs to the family of cellular receptor homologous to FcγRI. This receptor is expressed on memory B cells found on mucosal lymphoid tissues[125,126] and has an inhibitory potential[127]. Its expression was shown to be increase in peripheral B cells of HIV-infected viremic individuals[128]. Interestingly,

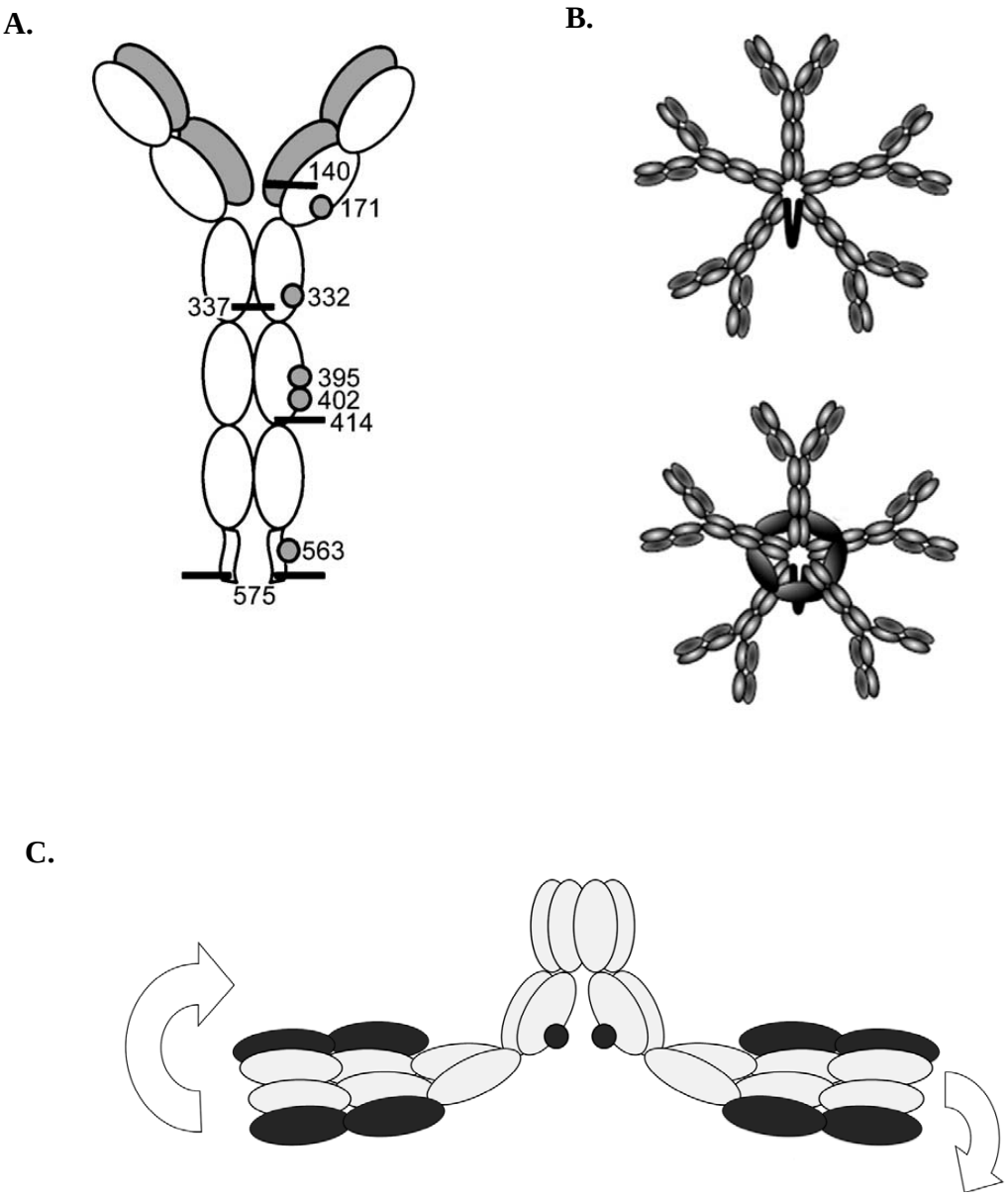

Figure 19: Structure of one IgM subunit, pentameric IgM, SIgM and spatial disposition of pentameric IgM. A. The figure represents an IgM subunit. Heavy chain domains are represented by white ovales and light chain domains are represented by grey ovales. Black lines and associated numbers indicate positions of cystein residues and disulphide bonds. Grey circles and associated numbers indicate positions of glycosylated asparagin residues. B. Pentameric IgM (up) and SIgM (down) C. The figure shows the spatial disposition of pentameric IgM as proposed in Czajkowsky et al., 2009[136]. Two subunits are represented. Heavy chains are indicated by light grey ovals and light chains are shown by dark ovals. Small dark circles represent the localization of C1q binding sites (adapted from Klimovich, 2011)[129].

Wilson et al. (2012)[130] identified that FcRL4 was able to bind IgA. The importance of this binding in B cell regulation remains poorly understood.

Other receptors

IgA receptors were described on human natural killer cells[131] and a particular kind of pIgR found on a murine B cells lymphoma was also identified. This latter receptor is able to preferentially bind IgM over IgA in a J chain-dependent manner[132].

4. Immunoglobulin M

IgM is a crucial Ab as it is the first to appear in organism development and it is the first to be produced by humoral immune responses[133]. IgM is found as a membrane-bound form on B cells but also secreted in blood and at the level of mucosal surfaces. At this level, IgM is present as SIgM[134].

4.1 Structure

Similarly to other Ab classes, the monomeric IgM molecule of 180 kDa is composed of two heavy and two light chains. The light chains are constituted of two domains (VL, CL), whereas the heavy μ-chains are constituted of one variable domain (Vμ) and four constant domains (Cμ1-4) with a tailpiece of 18 amino acids linked to the Cμ4 domain. Mu-chains carry five regions of N-glycosylation. Oligomannose glycans are present at positions Asn 402 and 563 and more complex glycans are attached to Asn 171, 332 and 395. Seven to twelve per cent of the molecular mass of IgM is due to glycosylation [135]. A disulfide bond is present between the Cμ1 domain (Cys 140) and a light chain, as well as between both Cμ2 domains (Cys 337) (**Figure 19 A**). IgM is present either as pentamer (900 kDa) (**Figure 19 B, up**), the predominant form found in blood and the only form found at mucosal surfaces, or hexamer. Subunits are associated together by cystein residues. S-S bridges are localized between the tailpieces (Cys 575) of subunits and Cμ3 domains (Cys 414) of neighboring monomers. The J chain only present in pentamers, is incorporated between two subunits and also forms a bond with Cys 575 residues[129,136,137].

The structure of pentamers was first described as a planar star-shaped complex[138,139]. However, a more recent model described a non-planar, mushroom-shaped structure with the C-terminal regions that protrude out from the plane constituted by the Fab-regions and Cμ2 domains[136] (**Figure 19 C**).

SIgM found at mucosal surfaces is composed of pentameric IgM and SC (**Figure 19 B, down**). As for SIgA, SC is bound to IgM during transcytosis into the luminal compartment via pIgR. IgM and SC are bound by covalent and noncovalent interactions[140,141]. Binding of IgM especially depends on strong interactions with domain D1 of SC[142] but Prinsloo et al., (2009)[143] also pinpointed the role of DII-V for high affinity IgM binding.

4.2 Roles of IgM in infections

IgM were shown to play a role in protection against infections[134] and development of secreted IgM-deficient mice largely contributed to understand this role[144,145]. However, models of infections aiming at testing the role of IgM are often models of systemic infections, instead of mucosal infections. Boes et al., (1998b)[146] demonstrated that secreted IgM-deficient mice were more susceptible to acute septic peritonitis induced by cecal ligation and puncture but their resistance was restored by administration of polyclonal IgM from wild type mouse serum. Along the same line, IgM exhibited protective effects against *Nocardia brasiliensis*[147], contributed to resolve *Borrelia hermsii* infection[148] and provided protection against *Streptococcus pneumonia* via the activation of complement[149]. The level of serum IgM also seems to play a role in protection against *Pseudomonas aeruginosa*[150]. Moreover, IgM plays a role in protection against intranasal infection by influenza virus[151,152,153]. Secreted IgM-deficient mice are more susceptible to West Nile Virus infection and intraperitoneal administration of polyclonal anti-West Nile Virus IgM protected them[154]. Finally, the role of IgM Abs was demonstrated in protection against fungus and parasite infections. The absence of serum IgM increased the susceptibility of mice to pulmonary challenge with *Cryptococcus neoformans*[155]. Couper et al., (2005 and 2005b)[156,157] revealed that immune IgM allowed to limit *Toxoplasma gondii*-dissemination and to restrain *Plasmodium chabaudi*-replication. And Baral et al., (2007)[158] described a control of *Trypanosoma evansi* infection mediated by IgM.

Pre-clinical studies have also investigated the efficacy of monoclonal IgM Abs against bacterial or parasitic infections. Passive immunization with a human monoclonal IgM reduced bacteremia and inflammation in a mouse model of systemic pneumococcal infection[159]. Another study showed that a monoclonal IgM Ab against *Pseudomonas aeruginosa* LPS provided protection in a murine pulmonary infection model[160]. Finally, Nouir et al., (2012)[161] demonstrated that passive administration of a monoclonal IgM Ab specific for *Strongyloides ratti* heat shock protein 60 protected mice against challenge infection. In these three studies, IgM was administered by intraperitoneal route.

A few pre-clinical and clinical studies reported effects of passive administration of polyclonal IgM-enriched preparations in case of sepsis. Using an acute respiratory distress syndrome rat model, Lachmann et al., (2004)[162] demonstrated that intravenous (i.v.) administration of IgM-enriched preparations reduced *Klebsiella pneumonia* infection. Some improvements were also observed in a rabbit model of sepsis induced by *E. Coli*[163]. Contrasting results were nevertheless obtained in clinical trials, possibly due to the study design and microbiological aetiology[164]. Some studies indicated non-significant results[165,166], whereas other ones showed significant recovery of the infected subjects. IgM-enriched preparation therapy led to a decrease of mortality in neonatal sepsis[167] and neonate short-term mortality[168], while Schedel et al. (1991)[169] showed a reduction of mortality in patients with endotoxin-positive septic shock. Behre et al. (1992)[170] also suggested a potential effect of i.v injection of IgM-enriched preparations with a decrease in endotoxin levels in plasma within the initial treatment period. Jackson et al. (1993)[171] examined the effect of IgM-enriched preparations used as prophylactic agent. The authors showed a significant reduction in endotoxaemia in bone marrow transplant patients. In order to dissect the mechanisms of these enriched preparations, a study compared the neutralization effects of IgM-, IgA and IgG-enriched preparations on streptococcal antigens. The authors concluded that IgM- and IgA-enriched preparations were the most potent inhibitors of specific streptococcal antigens[172]. Interestingly, two

clinical studies tested the use of IgM-enriched IVIg as additional treatment in the case of Crohn's disease. Even though the results of the first one were moderate[173,174], the second study[174,175] showed a marked improvement in Crohn's disease activity index in patients who received these preparations compared to the control group.

The mechanisms of protection conferred by monoclonal and polyclonal IgM injected via systemic route are not completely understood. Features of antigen neutralization and agglutination were naturally evoked but its capacity to activate the complement cascade was also mentioned. Modulation of Fc receptor expression, cytokine responses, immune cell functions and inflammation could be also involved[129,134,164,176].

4.3 IgM receptors

Polymeric immunoglobulin receptor (pIgR)

As previously mentioned, pIgR can bind pentameric IgM. The presence of the Cμ4 domain of IgM and J chain were shown to be crucial for pIgR binding[129].

Fcα/μ receptor (Fcα/μR)

As previously described, Fcα/μR is able to bind IgM. The Cμ3 and 4 domains of IgM seem to be essential for the interaction with the receptor[113]. However, in contrast to pIgR, the binding of IgM to Fcα/μR does not require J chain[177] and interestingly, the site of IgA-or IgM-binding on human Fcα/μR is thought to be in the same region[111,113].

Fcμ receptor (FcμR)

Kubagawa et al. (2009)[178] identified a complementary DNA that encoded a bona fide FcμR in human B-lineage complementary DNA libraries. FcμR is a transmembrane protein of ca. 60 kDa expressed on B and T cells. This receptor is an extracellular Ig-like domain homolog to pIgR and Fcα/μR but it binds only IgM.

5. Polyclonal immunoglobulin-based therapy

Polyclonal immunoglobulin preparations were prepared as a prophylactic treatment against measle infections 60 years ago[179]. Next, these preparations were used to treat immunodeficient patients[180] in order to prevent microbial infections during their life[181]. For numerous years, immunoglobulin therapies mainly based on IgG IVIg preparations, have been used with improvement of a large panel of additional disease conditions including autoimmune diseases, neurologic disorders, asthma, infectious diseases or even following transplantations[182]. In the context of intestinal diseases, a study described resolution of severe *C. difficile* diarrhea in 64% of elderly patients treated with IVIg who were refractory to antibiotic therapy[183]. In addition, a few studies reported significant improvements of Crohn's disease by IVIg administration with a drop of symptoms and an improved Crohn's disease activity index[174].

5.1 IVIg preparations

IVIg preparations consist mostly of IgG molecules that are purified from a pool of human plasma obtained from healthy donors (3,000-10,000 donors). Healthy people are by essence exposed to a panel of infections and vaccines and consequently, the nature of the Ab molecules they produce covers a broad range of specificity i.e are polyclonal[181]. IgG fraction is mainly obtained by ethanol precipitation and various steps of viral clearance[184]. Depending on the preparations, varying amounts of IgG aggregates are present but are in general removed to limit the risk of cytokine storm mediated via activation of Fcγ receptors[181].

5.2 Modes of action

Numerous modes of action have been described for IVIg. The $F(ab')_2$ region is able to neutralize cytokines, elements of complement and autoantibodies. In addition, via this region, IgG molecules are also able to block cell-cell interactions and to kill target cells via antibody-dependent cytotoxicity. The Fc

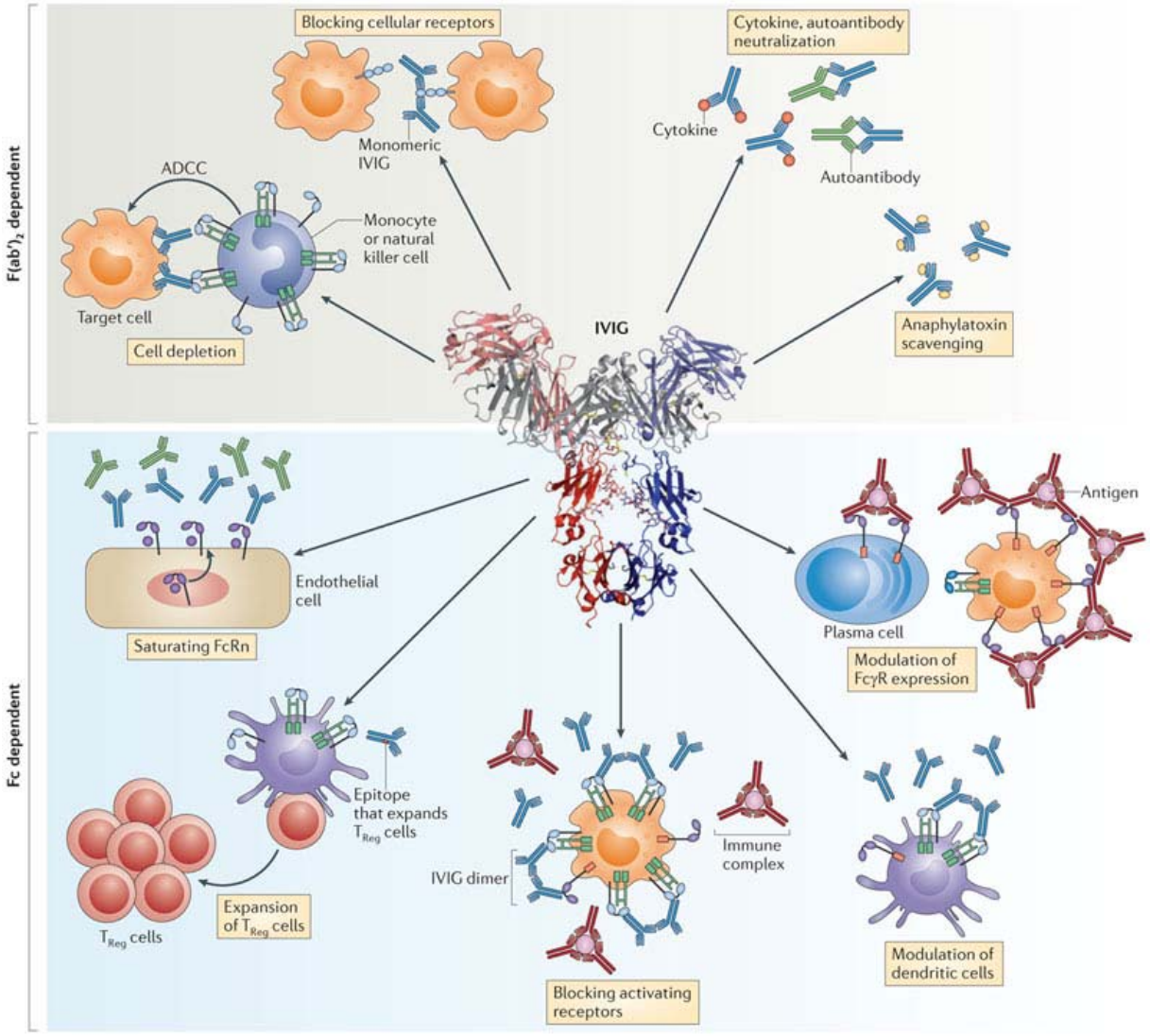

Figure 20: Modes of action of IVIg mediated by F(ab')$_2$ and Fc-dependent pathways. F(ab')$_2$-dependent pathways include: neutralization of cytokines and autoantibodies; blockade of cell-cell interactions mediated by cell-surface receptors; scavenging of complement elements (anaphylatoxins C3a and C5a); killing of target cells by antibody-dependent cytotoxicity (ADCC). Fc-dependent pathways include: saturation of the neonatal Fc receptor (FcRn); blockade of immune complex binding to Fcγ receptors (FcγRs); modulation of DC activation via FcγR; modulation of activating and inhibitory FcγR expression on innate immune and B cells; effect on expansion of Treg cells (adapted from Schwab and Nimmerjahn, 2013)[181].

region is able to associate with FcγR expressed on macrophages, monocytes, polymorphonuclear cells and DCs and therefore block immune complex binding, modulate DC activation and modulate activating or inhibitory FcγR expression on innate immune and B cells. The neonatal Fc receptor (FcRn) can be satured by IVIg administration and regulatory T cell expansion can be affected[181]. In case of infections, direct interactions with pathogens leading to activation of complement or Fc-mediated phagocytosis by antigen-presenting cells have been also described[185] (**Figure 20**).

5.3 Route of administration, doses and side effects

Igs were first intramuscularly administered but the risk of sometimes fatal systemic adverse reactions led to consider i.v administration in eighties. To further decrease the risk of adverse reactions, the possibility to inject Igs by subcutaneous route has been also examined[179]. The first study of SCIg therapy was reported in 1991[186], and less systemic adverse effects and a better maintenance of IgG levels were observed. The low rates of systemic reactions were confirmed in additional studies[187,188]. The results of these studies encouraged the use of SCIg. An additional advantage of SCIg is the possibility of self-infusions. This could increase the compliance of patients and reduce healthcare costs[189].

The dose and volume of injected Igs, as well as infusion intervals vary according to the individuals and the clinical context. However, most national and international guidelines recommend a starting dose of 0.4 g/kg per month for IVIg and SCIg[179].

The most frequent side effects are headaches, fever and nausea. Severe adverse reactions such as anaphylaxis or acute cardiovascular events are rare (<5% of patients treated with IVIg) [181,185,190] .

Aims of this work

IVIg therapy based on IgG Abs leads to significant clinical benefits. Indeed, IgG supplementation prevents important systemic infections in immunodeficient patients. However, despite IgG supplementation, they often experience recurrent airway infections[191] and gastrointestinal infections or inflammations[192]. On the one hand, animal studies have shown that mucosal application of pIgA or SIgA could prevent, diminish or even cure bacterial and viral infections[46]. In addition, demonstrated anti-inflammatory effects of IgA might also play an important role in the treatment of chronic mucosal infections and inflammations[193]. However, IgA/SIgA has been only used occasionally for therapeutic application. On the other hand, administration of polyclonal IgM-enriched preparations via the systemic route provided protection against infections in preclinical and clinical studies. However, potential benefits of polyclonal purified IgM/SIgM administered by mucosal route have never been studied.

The aim of this thesis project has been to determine if polyclonal human plasma-derived IgA and IgM can be used as a source of Abs to generate functional secretory-like IgA and IgM molecules, respectively. These molecules could be delivered to mucosal surfaces by passive immunization in order to fight a large panel of mucosal infections.

First, the work aimed at determining if IgA and IgM could be converted into secretory-like IgA and IgM molecules by association with SC and at establishing if these molecules had the same biochemical features as mucosa-derived Abs. Second, the functionality and the modes of action of human plasma IgA and IgM, as well as secretory-like IgA- and IgM molecules against a virulent strain of *S. flexneri* were examined using an *in vitro* model mimicking the GI epithelial barrier. Additionally, the functionality of human plasma IgA against another type of antigen, *C. difficile* toxin A, was also explored.

Part I: Human plasma-derived polymeric IgA and IgM antibodies associate with secretory component to yield biologically active secretory-like antibodies

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Overview of this part

This work aimed at evaluating the biochemical and functional characteristics of human plasma-derived IgA and IgM. First, we confirmed the presence of expected heavy and light chains, as well as the J chain in IgA and IgM-enriched preparations containing a mixture of various molecular forms of IgA and IgM, respectively. The presence of the J chain indicated that polymeric molecular forms were found in the preparations. Knowing that the association with SC is essential for a potential mucosal application, we demonstrated the specific interaction between recombinant human SC (hSC) and pIgA or IgM present in IgA- or IgM-enriched preparations, respectively. In order to evaluate more finely the association, pIgA was separated from mIgA, and IgM was purified by size-exclusion chromatography. The covalent interaction between purified pIgA or IgM and hSC was confirmed, as well as the 1:1 stoichiometry of association. As it had been already shown with IgA from other sources, we confirmed that the association between purified pIgA or IgM and hSC increased the stability of the Ab in the presence of intestinal proteases. Finally, we demonstrated the protective effect of pIgA and SIgA-like molecules using an *in vitro* model of reconstituted intestinal epithelial Caco-2 cell monolayers infected with a pathogenic strain of *S. flexneri*. As plasma-derived Abs are intrinsically polyreactive, these results open the way to assess their protective abilities against a large panel of pathogens using *in vitro* and *in vivo* models of infection.

Experimental procedures, results, discussion

The results obtained in this part are compiled in the paper published in the Journal of Biological Chemistry and entitled:

Human plasma-derived polymeric IgA and IgM antibodies associate with secretory component to yield biologically active secretory-like antibodies. Longet S (see author contribution), Miled S, Loetscher M, Miescher SM, Zuercher AW, Corthésy B. J Biol Chem. 2013 Feb 8;288(6):4085-94.

Author contribution

I have been involved in experiments dealing with biochemical analyses of antibodies, as well as in culture, stimulation, observations of cell monolayers and related analyses.

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Human Plasma-derived Polymeric IgA and IgM Antibodies Associate with Secretory Component to Yield Biologically Active Secretory-like Antibodies*

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Background: Production of SIgA or SIgM for therapeutic application remains an unsolved issue.
Results: Human plasma-derived polyclonal, polymeric IgA and IgM associate with recombinant or colostrum-derived human secretory component to form digestion-resistant, functionally active SIgA- and SIgM-like molecules.
Conclusion: SIgA and SIgM can be rebuilt *ex vivo* from plasma-derived IgA/IgM.
Significance: This would enable development of SIgA/SIgM-based mucosal therapeutics.

Immunotherapy with monoclonal and polyclonal immunoglobulin is successfully applied to improve many clinical conditions, including infection, autoimmune diseases, or immunodeficiency. Most immunoglobulin products, recombinant or plasma-derived, are based on IgG antibodies, whereas to date, the use of IgA for therapeutic application has remained anecdotal. In particular, purification or production of large quantities of secretory IgA (SIgA) for potential mucosal application has not been achieved. In this work, we sought to investigate whether polymeric IgA (pIgA) recovered from human plasma is able to associate with secretory component (SC) to generate SIgA-like molecules. We found that ~15% of plasma pIgA carried J chain and displayed selective SC binding capacity either in a mixture with monomeric IgA (mIgA) or after purification. The recombinant SC associated covalently in a 1:1 stoichiometry with pIgA and with similar efficacy as colostrum-derived SC. In comparison with pIgA, the association with SC delayed degradation of SIgA by intestinal proteases. Similar results were obtained with plasma-derived IgM. *In vitro*, plasma-derived IgA and SIgA neutralized *Shigella flexneri* used as a model pathogen, resulting in a delay of bacteria-induced damage targeted to polarized Caco-2 cell monolayers. The sum of these novel data demonstrates that association of plasma-derived IgA or IgM with recombinant/colostrum-derived SC is feasible and yields SIgA- and SIgM-like molecules with similar biochemical and functional characteristics as mucosa-derived immunoglobulins.

Mucosal surfaces of the digestive, respiratory, and urogenital tracts, as well as the ducts of exocrine glands are lined by layers of epithelial cells that form a tight barrier separating the internal compartments of the body from the outside environment. In humans, these vast surfaces cover 400 m^2, an area that is permanently exposed to exogenous pathogens (1). The combination of innate and inducible cellular and molecular mechanisms ensures protection against colonization and entry/invasion by microbes (2). In healthy individuals, secretory IgA (SIgA)[3] is the most abundant antibody (Ab) fulfilling the function of immune exclusion on the luminal side of mucosal surfaces (3), whereas secretory IgM (SIgM) Abs take over in IgA-deficient patients. To exert their specific protective function in mucosal secretions, polymeric IgA (pIgA) and IgM produced at effector sites are transported across the epithelium by the polymeric immunoglobulin receptor (pIgR) expressed on the basolateral side of epithelial cells (4). During transport, the pIgR is proteolytically cleaved, and the extracellular portion of the molecule, referred to as the secretory component (SC), is released in association with pIgA and IgM to form SIgA and SIgM (5, 6). Both secretory Abs are thus essential to maintain epithelial integrity.

As an alternative to vaccination, protective levels of Abs might directly be delivered to mucosal surfaces by passive immunization. In nature, this occurs physiologically in many mammalian species by transfer of maternal Abs to their offspring via milk (7, 8). Human and animal studies dealing with passive mucosal immunization have demonstrated that pIgA and SIgA Ab molecules administered by oral, intranasal, intrauterine, or lung instillation can prevent, diminish, or cure bacterial and viral infections (9). However, the secretory form naturally found at mucosal surfaces was rarely used, and purification/production of SIgA still remains a challenging task (10). In this study, we sought to determine whether polyclonal plasma-derived pIgA and IgM Abs can be transformed into secretory-like Abs *in vitro*. We found that IgA- and IgM-en-

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[3] The abbreviations used are: SIgA, secretory IgA; pIgA, polymeric IgA; SC, secretory component; mIgA, monomeric IgA; Ab, antibody; SIgM, secretory IgM; pIgR, polymeric immunoglobulin receptor; hSC, human SC; hSCrec, recombinant hSC; hSCcol, colostrum-derived hSC; mSC, mouse SC; TER, transepithelial electrical resistance; LB, Luria-Bertani; LSCM, laser-scanning confocal microscopy.

riched plasma preparations, or purified plasma pIgA and IgM, are able to associate with recombinant or colostrum-derived human SC (hSC). As in nature, disulfide bridges between hSC and the Abs formed with a 1:1 stoichiometry. Association with hSC delayed degradation of either pIgA or IgM upon exposure to intestinal washes rich in proteases. Biological activity of plasma-derived molecules was demonstrated in neutralization assays based on the protection of polarized Caco-2 epithelial cells used as a mimic of the intestinal epithelium against invasion by *Shigella flexneri*. Together, the data demonstrate that both reconstituted SIgA and SIgM display many biochemical features of secretory Abs and open the path to explore their protective function in *in vivo* models of infection.

EXPERIMENTAL PROCEDURES

Preparation of Human Plasma IgA- and IgM-enriched Fractions—IgA and IgM were purified from process intermediates of immunoglobulins manufactured from human plasma (11) by affinity chromatography using CaptureSelect Human IgA and CaptureSelect Human IgM resins (Bioaffinity Company BAC). Three different starting materials were used: 1) cryo-poor human plasma (termed "plasma"); 2) immunoglobulin-enriched cold ethanol precipitate (termed "paste"), a process intermediate obtained during large scale ethanol fractionation of human plasma proteins; 3) a chromatography side fraction (termed "column strip"), consisting of the strip fraction from an ion-exchange chromatography column used in the large scale manufacture of IgG from human plasma. The different starting materials were diluted in PBS to a target protein (IgA or IgM) concentration of ~1 mg/ml and then loaded onto a CaptureSelect Human IgA or IgM column pre-equilibrated with PBS, without exceeding the IgA- or IgM-binding capacity of the column. After loading, the column was washed with PBS, and IgA or IgM was eluted with glycine buffer at pH 3.0. The eluate was adjusted with 0.5 M Tris-base (pH 8.0) to pH 4.5 and concentrated up to 16 mg/ml protein.

Production/Purification of Recombinant Proteins and Colostral Human SC—Recombinant hSC (hSCrec) was produced from a CHO clone stably transfected with an expression cassette coding for the protein (12). Colostrum-derived hSC (hSCcol) was obtained as described (13). Mouse IgAC5 specific for *S. flexneri* LPS serotype 5a and recombinant mouse SC (mSC) were produced and purified as described (12, 14).

Western blot analysis—SDS-PAGE and transfer onto PVDF membranes was carried out as described (15). The membranes were then blocked for 30 min in PBS-0.05% Tween 20 solution (PBS-T) containing 1% BSA. Detection of the polypeptides in IgA- and IgM-enriched or purified IgA and IgM preparations was carried out with: 1) rabbit IgG anti-human alpha chain, HRP-conjugated (Dako, 1/5,000 dilution); 2) rabbit IgG anti-human mu chain, HRP-conjugated (Dako, 1:5,000 dilution); 3) goat anti-human kappa chain (Cappel, 1/3,000 dilution), followed by secondary anti-goat HRP-conjugated antiserum (Pierce, 1/5,000 dilution); 4) rabbit anti-J chain antiserum (1/3,000 dilution) (16), followed by secondary anti-rabbit HRP-conjugated antiserum (Sigma, 1/3,000 dilution). In reconstituted SIgA or SIgM, the presence of hSC was assessed using rabbit anti-hSC antiserum (1/3,000 dilution) (17), followed by secondary anti-rabbit HRP-conjugated antiserum (Sigma, 1/10,000 dilution). In reconstituted SIgAC5, the presence of mouse SC (mSC) was assessed using rabbit anti-mSC antiserum (1/3,000 dilution) (14), followed by secondary anti-rabbit HRP-conjugated antiserum (Sigma, 1/5,000). All incubations were performed in PBS-T containing 0.1% BSA at ambient temperature for 1–2 h. After final washing with PBS-T, immune complexes on membranes were detected by chemiluminescence and exposure on autoradiographic films.

Dot Blot Reassociation Assay—Dot blot reassociation assays were essentially carried out as described (17) with the following modifications: blotting membranes consisted of PVDF; blocking solution was PBS-T containing 1% BSA; IgA- and IgM-enriched preparations were used for overlay incubation in 200 μl of PBS-T containing 0.1% BSA; and detection Abs were directly coupled to HRP.

Separation of Plasma-derived pIgA and mIgA and Purification of Plasma-derived IgM—IgA-enriched preparations containing a mixture of mIgA and pIgA were diluted in PBS to a final volume of 10 ml suitable for injection onto the ÄKTA*prime* chromatography system (GE Healthcare). The flow rate was set at 1 ml/min with PBS as the mobile phase for all runs. To resolve the two molecular forms of IgA from other plasma components, the material was initially applied onto a 1-meter-long column filled with Superdex 200 resin (GE Healthcare). Separation of the two molecular forms of IgA was also performed on two serially coupled 1-meter-long columns filled with Sephacryl S-300 HR beads (18). The IgA content of 3.5-ml fractions was verified by immunodetection, and pools of mIgA and pIgA were obtained. IgM-enriched preparations run under identical conditions yielded a single fully excluded peak. Concentration was performed using the Labscale system (Millipore) connected to a 100-kDa cut-off cartridge and stored at 4 °C until further use.

In Vitro Association of Polymeric Ig and hSC—SIgA molecules were obtained by combining *in vitro* 10 μg of purified pIgA molecules with 2.5 μg of either hSCrec or hSCcol. SIgM molecules were obtained by combining *in vitro* 10 μg of purified IgM molecules with 1.5 μg of either hSCrec or hSCcol. SIgAC5 molecules were obtained by combining *in vitro* 10 μg of purified pIgAC5 molecules with 2 μg of mSC. Association was performed in PBS for 30 min at ambient temperature as described previously (19). Integrity and correct assembly of the molecules into possible covalent complexes was examined by SDS-PAGE under non-reducing and reducing conditions, followed by Western blotting and immunodetection with antiserum specific for SC as indicated above.

FPLC Sizing Column Chromatography—To further assess the assembly of purified pIgA with hSCrec or hSCcol, complexes with a 1:1 and 1:2 stoichiometry were prepared in a final volume of 100 μl (total amount of protein: 10 μg) and passed over a 1 × 30-cm Superose 12 HR prepacked column (GE Healthcare) hooked to the ÄKTA*prime* chromatography system at a constant flow rate of 0.2 ml/min. Co-elution of bound hSC with pIgA, reflecting covalent association, was verified by immunodetection specific for hSC, and quantification of IgA and hSC in pooled fractions was carried out by ELISA (20).

Digestion of Abs with Mouse Intestinal Washes—Collection of intestinal washes form BALB/c mice (4–6 weeks old) was done according to the published procedure (19). For *in vitro* digestion, 125 ng of purified pIgA and reconstituted SIgA, or purified IgM and reconstituted SIgM, were mixed or not with 1 or 2 μl of intestinal washes in a final volume of 20 μl of PBS and incubated at 37 °C for various periods of time. Reactions were stopped by the addition of 2 μl of Complete^{TM} protease inhibitor mixture (Roche Applied Science) and kept frozen prior to analysis by immunoblot detecting the reduced form of the heavy chain of the antibody.

Caco-2 Cell Culture and Growth as a Polarized Monolayer—The human colonic adenocarcinoma epithelial Caco-2 cells (American Type Tissue Collection) were grown in complete DMEM consisting of DMEM-Glutamax (Invitrogen) supplemented with 10% FCS (Sigma), 10 mM HEPES (Invitrogen), 1% non essential amino acids (Invitrogen), 1% sodium pyruvate (Invitrogen), 1% L-glutamine (Sigma), 1% penicillin/streptomycin (Sigma), and 0.1% transferrin (Invitrogen), and used between passages 32 and 40. Cells cultivated up to 80% confluency were seeded on polyester Snapwell filters (diameter, 12 mm; pore size, 0.4 μm; Corning Costar) at a density of 0.8×10^5 cells/cm^2. At week 3, the Caco-2 cell monolayer integrity was checked by measuring the transepithelial electrical resistance (TER) using the Millicell-ERS device (Millipore) (21). TER values of well differentiated monolayers were in the range of 380–550 ohms $\times$ cm^2.

Bacterial Strain and Culture Conditions—Bacteria used were the virulent strain of serotype 5a LPS *S. flexneri* M90T constitutively expressing GFP (22). Bacteria from frozen stock were grown in Luria-Bertani (LB) agar plate containing 0.1‰ Congo Red (Applichem) and 50 μg/ml ampicillin (Sigma-Aldrich), for 30 h at 37 °C. Three red colonies were amplified in 10 ml of LB liquid broth supplemented with 50 μg/ml ampicillin (LB liquid/ampicillin) for 16 h at 37 °C and 200 rpm. The culture was centrifuged at 2,000 $\times$ *g* for 5 min, resuspended in PBS, diluted 1/10 in 10 ml LB liquid/ampicillin and then cultured for 2 h at 37 °C with shaking (200 rpm). Finally, bacteria in the exponential phase were washed twice in PBS by centrifugation at 2,000 $\times$ *g* for 5 min and resuspended in PBS. Assessment of cfu/ml was carried out by measurement of the optical density at 600 nm with the knowledge that 1 optical density unit at 600 nm corresponds to 5×10^8 cfu/ml.

Incubation of Bacteria with Different Ab Preparations—2×10^7 bacteria were mixed with 0.049 μM of SIgAC5 specific for *S. flexneri* LPS serotype 5 or with human plasma-derived pIgA (0.61 μM), SIgA (0.61 μM), or mIgA (0.61 μM), respectively, in a final volume of 500 μl of plain DMEM (DMEM complemented with 10 mM HEPES (Invitrogen), 1% non essential amino acids (Invitrogen), 1% sodium pyruvate (Invitrogen), 1% L-glutamine (Sigma), and 0.1% transferrin (Invitrogen)). The mixtures were incubated for 1 h at ambient temperature under gentle agitation.

Protection Assay—1 h before the use of polarized Caco-2 cell monolayers, complete DMEM was replaced by plain DMEM in both the apical and basolateral compartments. Polarized Caco-2 cell monolayers were infected apically with *S. flexneri* serotype 5a alone or in combination with the Ab preparations. Exposure of Caco-2 cells to *S. flexneri* or the various immune complexes was performed for up to 13 h, and bacteria-induced damage was tracked by measuring TER decrease over time.

Laser-scanning Confocal Microscopy (LSCM) Observation of Caco-2 Cell Monolayers—To examine the integrity of Caco-2 cell monolayers, Snapwells were washed twice with PBS, prior to fixation overnight with 5 ml of 4% paraformaldehyde at 4 °C. After washing, filters were permeabilized, and nonspecific binding sites were blocked using PBS containing 5% FCS and 0.2% Triton X-100 for 30 min at ambient temperature. All Abs were diluted in PBS-T. Filters were incubated with rabbit anti-human Zonula occludens-1 (ZO-1) (1/200 dilution, Invitrogen) for 2 h at ambient temperature, washed in PBS, followed by goat anti-rabbit IgG conjugated with Alexa Fluor® 647 (1/100 dilution, Invitrogen) for 90 min at ambient temperature. To visualize cells, filters were finally incubated with 200 ng/ml of 4′,6′-diamidino-2-phenylindole (DAPI; Invitrogen) in PBS for 30 min. Filters were cut out of their holders and mounted in Vectashield solution (Vector Laboratories) for observation using Zeiss LSM 710 Meta confocal microscope (Carl Zeiss) equipped with a 40$\times$ objective. Three-dimensional reconstructions along the xy plans were performed with the Zeiss ZEN 2009 light software.

Quantification of the Infected Areas—Observation of whole filters was carried out with the 10$\times$ objective using Zeiss ZEN 2009 light software. The sum of infected areas was determined using the particle analysis tool of ImageJ software applied onto the channel associated with the bacteria.

Statistical Analysis—Results were expressed as means $\pm$ S.E. of the mean. Student's *t* test analysis was performed using GraphPad Prism software (version 5). Differences were considered as significant when $p < 0.05$.

RESULTS

Plasma-derived IgA and IgM Contain J Chain and Assemble as Polymers—Human blood is known to contain mostly mIgA, with a minor proportion of pIgA (12–15% range) (23). We first analyzed whether plasma-derived, polyclonal pIgA contained J chain, as this is a prerequisite for subsequent assembly with SC (24). Immunoblot analysis of various plasma IgA-enriched preparations confirmed that in addition to the major mIgA fraction, an SDS-resistant polymer-fraction was present that carried all three expected polypeptides, *i.e.* the α and κ chains, and the J chain (Fig. 1*A*). Interestingly, the immunoreactivity toward the J chain depended on the starting material used to obtain the IgA-enriched fractions, suggesting varying content of pIgA in the different source materials. Incorporation of J chain in monomeric forms resulting from partly covalently assembled pIgA was also observed (20). Most of the pIgA in all three preparations contained covalently bound κ chain. As expected, plasma IgM Abs contained J chain, a feature of the pentameric molecular form, as well as the μ and κ chains (Fig. 1*B*).

Plasma-derived IgA and IgM Both Bind to Immobilized hSC in Vitro—We have demonstrated previously that dot blot reassociation assay is an appropriate assay to test interaction of polymeric Abs with SC, even when relatively crude material is used (17). The multistep assay (Fig. 2*A*) was thus used to assess

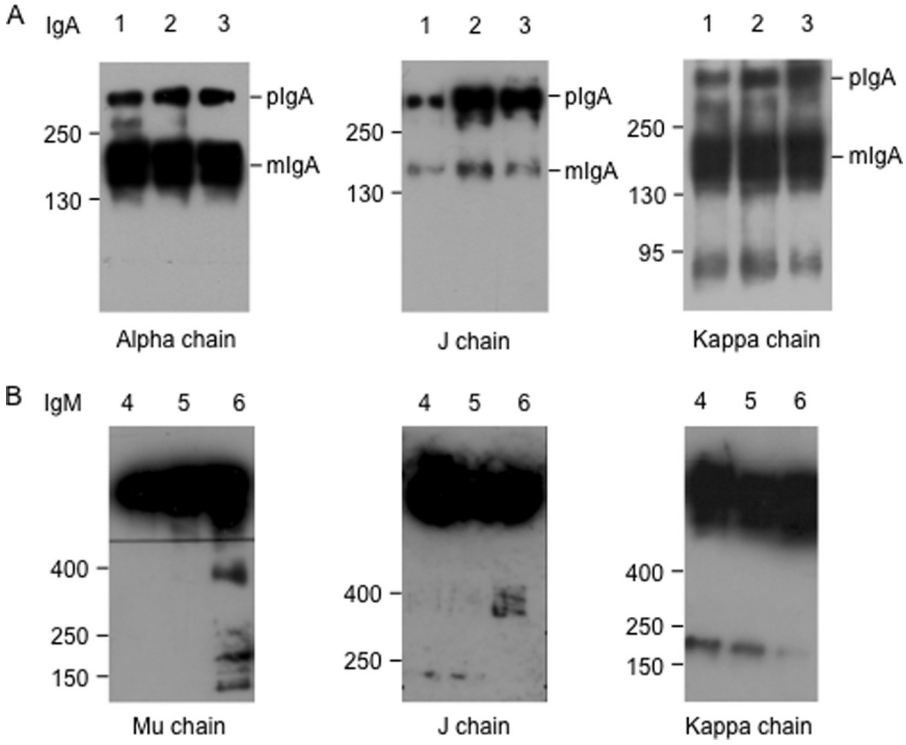

FIGURE 1. **Biochemical characterization of plasma IgA- and IgM-enriched preparations.** Immunoblot analysis under non-reducing conditions of IgA (*A*; *lanes 1–3*) and IgM (*B*; *lanes 4–6*) recovered from various IgA- and IgM-enriched preparations obtained from plasma (*1* and *4*), paste (*2* and *5*), or column strip (*3* and *6*) as described under "Experimental Procedures." The specificity of the antisera used is indicated *below* the respective panels. *A*, 200 ng of IgA was used for detection with anti-α chain antiserum, 500 ng for detection with anti-J chain antiserum, and 400 ng with anti-κ chain antiserum. *B*, 250 ng of IgM was loaded per lane, and signals were obtained using detection with anti-μ chain antiserum, and anti-J chain and κ chain antisera as for IgA. The position of migration of molecular weight markers is indicated alongside the panels.

the ability of purified hSCrec to bind with pIgA and IgM present in IgA- and IgM-enriched preparations, respectively. Immobilization of hSCrec on membranes followed by overlay incubation with preparations enriched in plasma IgA led to specific detection of IgA-positive signals only when all partners were incubated sequentially (Fig. 2*B*). The same was true when preparations enriched in plasma IgM were added in the overlay phase, yielding positive signals only in the presence of all components (Fig. 2*C*). In control experiments with human plasma-derived IgG, no signal was obtained, even at a 10-fold molar excess of the IgG or SC (data not shown). Binding to immobilized hSCcol yielded the same results, confirming the validity of hSCrec as a surrogate for the natural protein (data not shown) (25). Specific interaction between hSCrec or hSCcol and IgA- and IgM-enriched preparations prompted us to separate pIgA from mIgA and to purify IgM to more precisely analyze the association.

Fractionation of pIgA and mIgA Present in Plasma-derived Preparations—Size exclusion chromatography is a robust technique to separate pIgA from mIgA recovered from hybridoma cell supernatants (15) and from cell clones engineered to produce IgA (26). Two different resins and column sizes were used in the present study. Superdex 200 with a fractionation range of 10–600 kDa yielded several fractions containing J chain-reactive material indicative of the presence of pIgA (Fig. 3*A*). α chain-positive bands migrating at the position of mIgA co-eluted with pIgA, due to either insufficient resolution or the presence of non-covalently associated pIgA molecules that dissociate during non-reducing SDS-PAGE. To resolve this issue, Sephacryl S-300 HR (fractionation range, 10–1,500 kDa) was tested. J chain-positive pIgA was recovered in fractions in the first elution peak, whereas mIgA lacking J chain (data not shown) represented the major species in the second peak (Fig. 3*B*). Again, some apparent mIgA co-eluted with the pIgA fraction, likely representing non-covalently associated mIgA molecules that dissociated during SDS-PAGE. In support of this hypothesis, the non-covalent nature of human pIgA1 has been reported after analysis by SDS-PAGE under reducing and non-reducing conditions (27). In sizing chromatography run under native conditions, we found that the mixture of pIgA1 and pIgA2 naturally present in the plasma-derived preparations co-eluted.

Purified pIgA and IgM Assemble in Covalent Complexes with SC—In mucosal secretions and following *in vitro* association between monoclonal pIgA and hSC or mSC, SIgA exists as a covalent complex involving single disulfide bridges that can be identified by SDS-PAGE under non-reducing conditions. Equimolar amounts of purified pIgA and either hSCrec or hSCcol were allowed to associate for 30 min. They were then run on a denaturing polyacrylamide gel, transferred onto PVDF membranes, and immunodetected with anti-hSC antiserum. As for SIgA isolated from colostrum (13) and reconstituted recombinant SIgA Abs (15), we found partial covalency to occur, as indicated by the detection of SC signals at the position of migration of pIgA (Fig. 4*A*). Incubation of mIgA or IgG with hSC did not result in a covalent association, as there was no shift

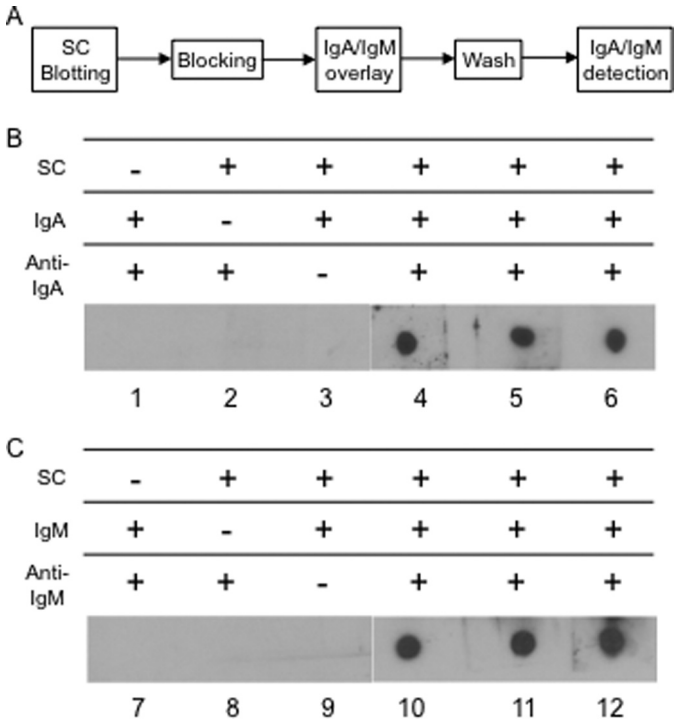

FIGURE 2. **Dot blot association assay of plasma-derived IgA and IgM with hSCrec.** *A*, schematic representation of dot blot reassociation assay. The successive incubation steps are depicted. *B* and *C*, binding of the various preparations enriched in plasma-derived IgA (*lanes 4–6*; *B*) and IgM (*lanes 10–12*; *C*) to immobilized hSCrec. *Lanes 4* and *10*, Ig from plasma; *lanes 5* and *11*, Ig from paste; *lanes 6* and *12*, Ig from column strip, as described under "Experimental Procedures." Control conditions (*lanes 1–3* and *7–9*) include lack of hSCrec ligand, lack of IgA or IgM, or lack of detection antiserum.

in position of hSC on the blot (data not shown). The same analysis of purified IgM associated with hSC resulted in the formation of covalent SIgM (Fig. 4*A*). This confirmed that pentameric IgM containing J chain is the major molecular form of the Ab in plasma (28). Of note, the immunoreactivity of bound hSC in SIgA is known to be strongly reduced, and thus, the true percentage of covalent binding is much higher than it appears by hSC-specific immunodetection (15). The involvement of disulfide bridges in covalent association between hSC and either IgA or IgM was confirmed by treatment with DTT, resulting in the detection of free hSC only (Fig. 4*A*).

Stoichiometry of Association between hSC and Plasma-derived pIgA or IgM—One can argue that the partial covalency observed upon association of the IgA/IgM and hSC is due to a portion of binding-incompetent, possibly denatured, molecules. To exclude this hypothesis, reconstituted SIgA prepared from a 1:1 and 1:2 ratio of Ab to hSC was separated on a sizing column (fractionation range, 10–2,000 kDa) using fast-protein liquid chromatography (Fig. 4*B*). Determination by ELISA of the IgA content of the fraction yielded identical values for 1:1 and 1:2 associated preparations, indicating an equimolar stoichiometry of association (Fig. 4*C*). Use of a 2-fold molar excess of hSC resulted in the additional appearance of late-eluting free hSC (Fig. 4*B*, *inset*), further indicating a specific, saturable level of interaction. Both recombinant and colostrum-purified hSC exhibit the same binding properties (Fig. 4*C*) (19). Thus, incomplete covalent association in SIgA most likely reflects intrinsic properties of the proteins and not major differences between the natural hSCcol and recombinant hSC. This also demonstrates that plasma-derived pIgA display all of the necessary structural features to serve as a ligand to hSC. The same set of

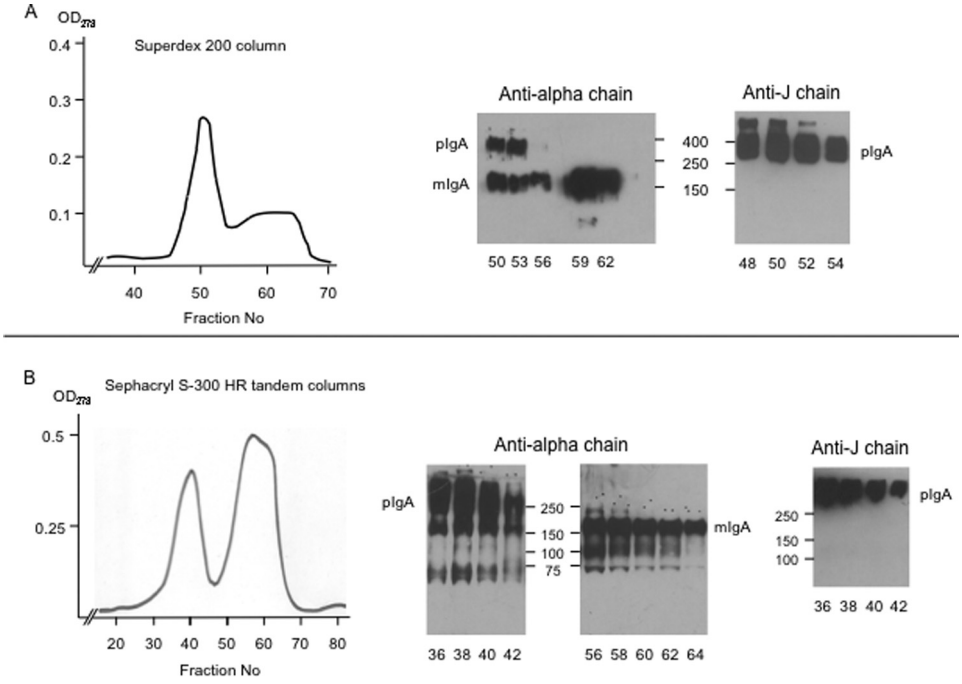

FIGURE 3. **Fractionation of polymeric *versus* monomeric plasma IgA.** *A*, elution profile of the 500-ml Superdex 200 column run in PBS. Immunoblot analysis of a selection of fractions confirmed the presence of J chain-containing pIgA (purified from column strip) in the first elution peak, whereas mIgA was eluted later. *B*, elution profile of the tandem (2 × 500-ml) Sephacryl S-300 HR columns run in PBS. Immunoblot analysis performed as for *A* demonstrates a better resolution of (J chain-containing) polymeric and mIgA. Pooled fractions of pIgA were used for *in vitro* association experiments with hSC.

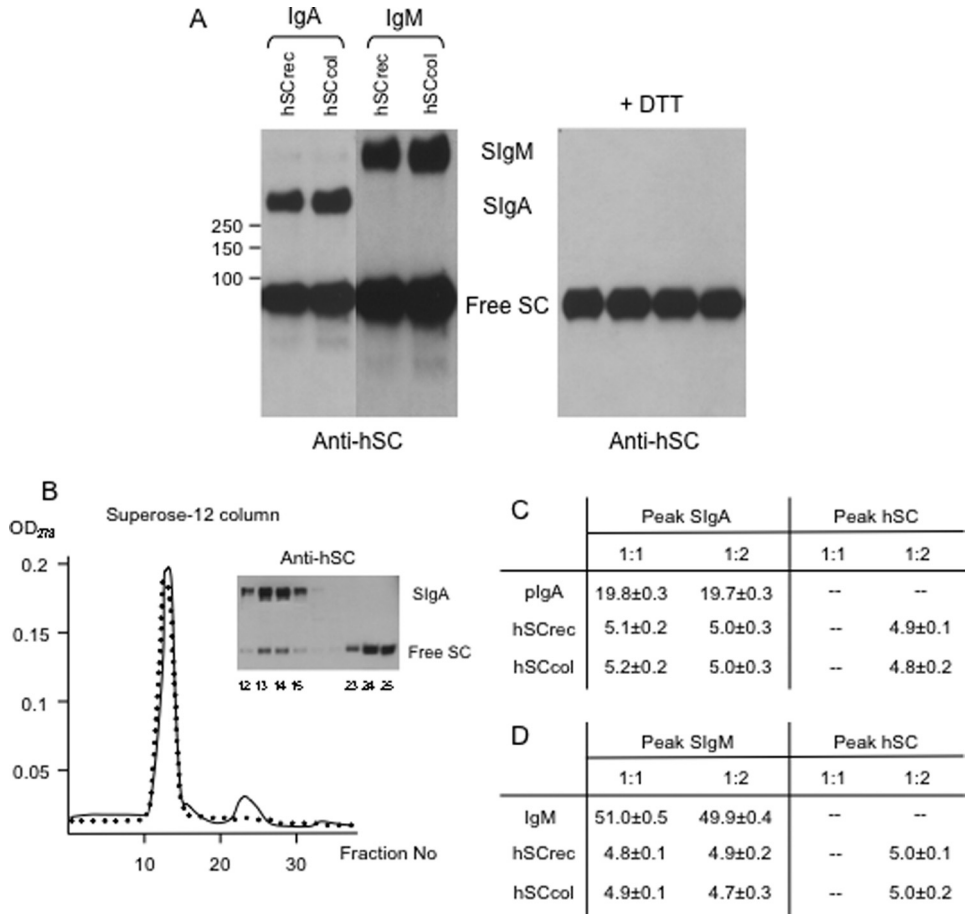

FIGURE 4. **Analysis of *in vitro* reconstituted SIgA and SIgM.** *A*, covalent association of purified pIgA and IgM (purified from column strip) with hSCrec and hSCcol. The formation of covalent complexes was detected by immunoblot using anti-hSC antiserum. After treatment of covalent complexes with the reducing agent DTT (+DTT) only the free hSC was detected on the immunoblot. The position of migration of molecular weight markers is indicated alongside the panels. *B*, elution profiles after molecular sieve chromatography in PBS solution of reconstituted SIgA in 1:1 (*dotted line*) and 1:2 (*black line*) ratio of pIgA to hSC; the *inset* shows immunodetection of hSC in a mixture at a 1:2 ratio, demonstrating the presence of co-eluting covalent and noncovalent hSC with pIgA. The excess of free hSC is eluted as an independent peak in late fractions. *C*, quantification by ELISA of pIgA, hSCrec and hSCcol (expressed in μg) in pooled fractions corresponding to peaks containing reconstituted SIgA or excess of free hSC. −, below level of detection.

experiments was carried out with reconstituted SIgM, yielding identical association results (Fig. 4, *A* and *D*).

Reduced Sensitivity of SIgA and SIgM to Proteases Indicates Correct Assembly as Secretory Abs—To test whether association of plasma pIgA or IgM with hSCrec would confer increased stability for potential mucosal application, the susceptibility of pIgA, SIgA, IgM, and SIgM to digestion with intestinal washes was examined as described previously (19). Changes in the migration profile of the α and μ heavy chains reflecting degradation were assessed by immunoblot after separation by SDS-PAGE under reducing conditions. Initially, conversion of the 62-kDa α chain into a band migrating at 40 kDa was observed at 2 h (Fig. 5*A*). This intermediate degradation product disappeared at 4 h, an effect due to the stringent conditions encountered in intestinal washes (19). At 6 h, most of the α chain in IgA was degraded, whereas the SIgA material displayed preserved integrity. In contrast to IgA, the SIgA counterpart survived overnight digestion (16-h time point, Fig. 5*A*). Generally, IgM appeared to be less sensitive to the action of intestinal washes (Fig. 5*B*). Nevertheless, as revealed in Fig. 5*B*, appearance of degraded mu chain fragments occurred more rapidly and more extensively for IgM compared with SIgM. To confirm this, densitometric analyses of the immunoblot films were performed. The sum of signals resulting from the degraded μ chain divided by the sum of signals corresponding to all immunoreactive species in the individual lane (×100) yielded the percentage reported in Fig. 5*C*. We conclude that although the phenomenon is not as marked as for SIgA, the sensitivity of SIgM to intestinal washes is reduced in comparison with IgM.

Human Plasma-derived IgA Protects Polarized Caco-2 Cell Monolayers from Damage by S. flexneri—To study the protective potential of human plasma-derived IgA, an *in vitro* model of reconstituted intestinal epithelial cell monolayers infected with a virulent strain of *S. flexneri* was used. The advantage of this model is that it reflects epithelial cell infection resulting from exposure to bacteria, viruses, and bacterial toxins and has proven very valuable to examine the neutralizing properties of Abs of various isotypes and in particular IgA (26, 29–31). The extent to which plasma-derived polyclonal mIgA, pIgA, or SIgA confer protection of epithelial cells was evaluated in comparison with an anti-*Shigella* protective mAb (IgAC5). TER values, LSCM observations, and quantification of infected areas were independently assessed to determine the integrity of Caco-2 cell monolayers. TER reflects the increased passage of ions and

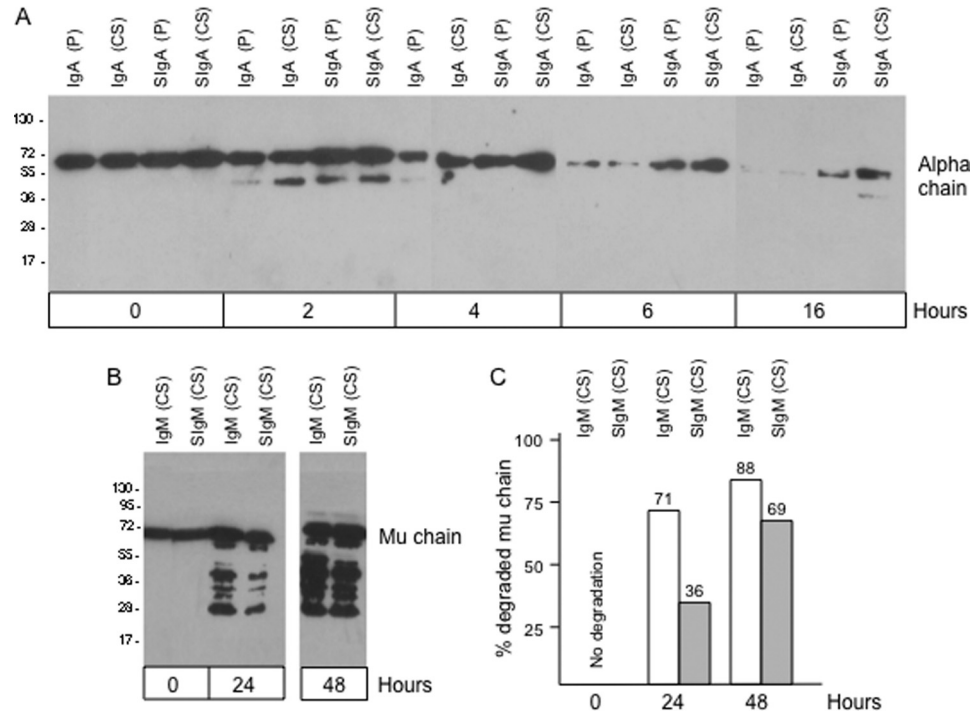

FIGURE 5. **Pattern of digestion of pIgA, SIgA, IgM, and SIgM incubated with intestinal washes.** Immunoblot analysis under reducing conditions of various preparations of purified IgA (purified from paste (*P*) or column strip (*CS*) as indicated in the figure; *A*) and IgM (purified from column strip; *B*) left as such or reconstituted into secretory Ab exposed to intestinal washes for increasing periods of time. Disappearance of α chain and degradation of μ chain reflects the action of proteases in the intestinal washes. The nature of digested proteins and the time course of incubation are indicated *above* the lanes. The position of migration of molecular weight markers is indicated alongside the panels. *C*, percentage of degraded μ chain in *B* analyzed by densitometry of the immunoblot. Calculated values are indicated on the *top* of columns.

indirectly damage to the epithelial monolayer. A similar weak reduction in TER was measured when either the IgAC5 mAb or a 10- to 12.5-fold higher concentration of plasma-derived pIgA or SIgA were examined, whereas bacteria alone or in combination with mIgA led to a marked drop within 13 h (Fig. 6*A*). Additionally, LSCM observations were performed 13 h post-infection. *S. flexneri* alone induced extensive damage reflected by widespread areas of the Transwell membrane devoid of cells (Fig. 6*B*, representative pattern). Maintenance of the cell monolayer integrity with human plasma pIgA and SIgA was very close to that obtained with SIgAC5, with occasional holes forming (Fig. 6*B*, representative pattern). Strikingly, mIgA does not offer the same degree of protection (Fig. 6*B*). In a more quantitative approach, the sum of infected areas of all sizes was determined for each individual whole filter ($n = 4$) at 13 h and is expressed as the surface (in mm^2) devoid of organized Caco-2 cell monolayers (Fig. 6*C*).

DISCUSSION

For IgA Abs to be effective upon mucosal application, the association with SC is essential (9). In the airways and the gut, SC-mediated anchoring to mucosal epithelial cells ensures optimal protective function (12), whereas in the intestine, SC confers improved stability to the IgA molecule (19). Purification of SIgA from a natural source, such as milk, intestinal fluid, or saliva is possible at small scale, but these materials are not appropriate sources for IgA/IgM-production on a larger scale. The possibility to associate plasma-derived pIgA Abs with hSCrec as described in this work indicates that it might be feasible to develop a plasma IgA-based product for mucosal application.

The detection of low levels of IgA monomers by SDS-PAGE in the plasma-derived pIgA fraction after separation by size exclusion chromatography suggests that a portion of pIgA is assembled as a non-covalent species sensitive to the presence of the detergent. This material is devoid of J chain and has been reported in analyses of hybridoma cell culture supernatants (15), in samples of bile and feces (16), in CHO cell line clones expressing pIgA (20), and even in human colostrum (13).

One can argue that due to different environments of biosynthesis for mucosal and serum IgA, the structure of the polymeric form may be different, resulting in a different capacity to recognize pIgR/SC *in vivo* and *in vitro*. However, the fact that hSCrec and "natural" hSCcol both effectively associate with plasma pIgA indicates that existence of intrinsic conformational plasticity in interacting partners is adequate to promote *in vitro* reconstitution of SIgA. This is in keeping with the delivery of pIgA in the mouse circulation, which allows the recovery of SIgA in secretions (32). We have established that covalent, disulfide bridge-mediated binding between plasma pIgA and hSC occurs, further demonstrating the efficiency of the *in vitro* assembly process. Similar results were observed for SIgA recovered from human colostrum (13, 33) and may be due to the reduced capacity of SC in SIgA2 to form covalent complexes, as compared with SIgA1 (27). Alternatively, in rodent species with a single IgA subtype, partial covalency is observed systemati-

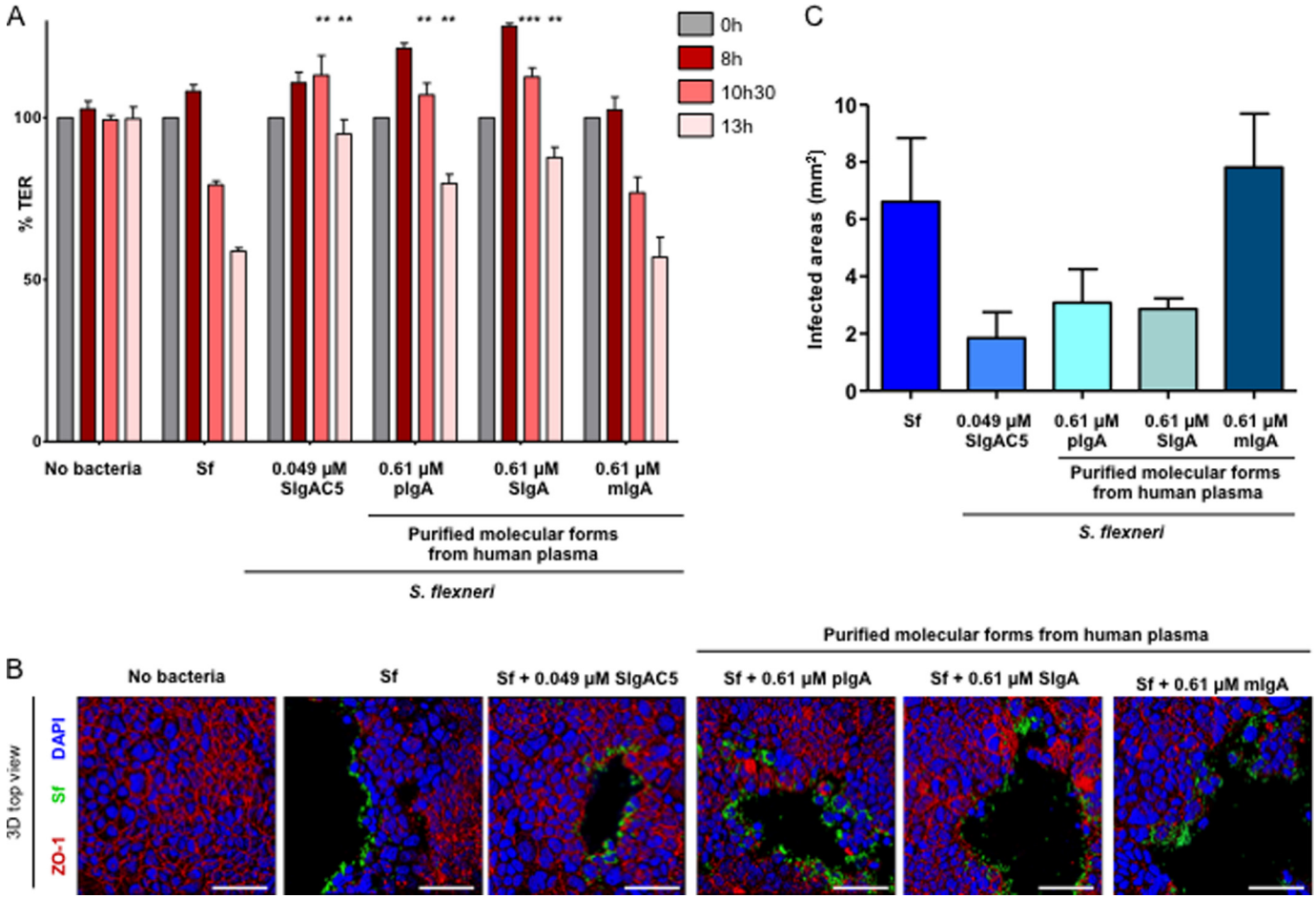

FIGURE 6. **Integrity of Caco-2 cell monolayers infected with *Shigella flexneri* alone or in combination with various IgA preparations.** *A*, TER of intestinal Caco-2 cell monolayers exposed to 2×10^7 *S. flexneri* M90T alone or in combination with purified column strip-derived pIgA, SIgA, or mIgA, determined at four time-points. The TER values for each condition and each time point were normalized with the TER values at the beginning of the experiment and are expressed in percentage. Protection offered by SIgAC5 specific for *S. flexneri* LPS and non-infected Caco-2 cell monolayers (no bacteria) serve as controls. The panel is representative of one individual triplicate experiment performed three times. Significant statistical differences calculated by comparison with the condition *S. flexneri* alone (*Sf*) are indicated above the columns: **, $p < 0.01$; ***, $p < 0.001$. *B*, LSCM three-dimensional reconstructed images (snapshot) of Caco-2 cell monolayers exposed to 2×10^7 *S. flexneri* M90T alone or in combination with human plasma-derived pIgA, SIgA, or mIgA for 13 h. Tight junctions stabilizing the monolayer are visualized with ZO-1 labeling (*red*). Caco-2 cells are visualized via nuclear staining with DAPI (*blue*) and bacteria constitutively expressing GFP stain (*green*). Control monolayers are the non-infected Caco-2 cell monolayers (no bacteria). *Scale bars*, 50 μm. *C*, for each condition, the sum of infected areas was determined from LSCM pictures of whole filters using ImageJ software. *B* and *C* are representative of two independent experiments performed in duplicates.

cally (34) and may reflect intrinsic exchange between disulfide bonds in the α chain and domain 5 in SC (19).

In secretions, only pentameric, J chain-containing IgM is released as SIgM. It is therefore highly likely that this molecular form in blood is responsible for the interaction we have detected with hSC. Our data are the first to show that partially covalent, disulfide bridge-mediated interaction indeed takes place between IgM and hSC. Reconstitution as SIgM allowed us to address the positive impact SC has on the stability of SIgM when exposed to proteases. Hence, the "sacrificial" transport of IgM by pIgR may be similarly beneficial to the half-life of the Ab, as already known for SIgA. In conclusion, although it has never been formally demonstrated that pIgR and SC bind identically to pIgA/IgM (35), and despite the presence of mIgA in the plasma preparation, *in vitro* association with SCrec and SCcol is able to take place with both IgA and IgM.

The sum of these data indicates that essential biochemical properties, including J chain-dependent binding specificity to SC (recombinant as well as colostrum-derived), increased stability in protease-rich intestinal washes, and covalent association occur after association between SCrec/SCcol and plasma-derived IgA/IgM. In addition, the remarkable protective potential against *S. flexneri* with human plasma-derived reassociated SIgA but also pIgA using an *in vitro* model of intestinal Caco-2 cell monolayers shows that the polyreactive Abs are functional. Remarkably, only a 10- to 12.5-fold higher concentration was required to reach similar degrees of protection as the specific SIgAC5 mAb via a mechanism of action relying on Ab-mediated bacterial aggregation. Both pIgA and reconstituted SIgA exhibit a similar neutralizing capacity at concentration (0.61 μM) close to that measured in human gut lavage fluids (36), thus opening up a therapeutic possibility for passive immunization with the optimal molecular form, *i.e.* reconstituted SIgA. As plasma-derived Abs are intrinsically polyreactive, future work will evaluate their protective capabilities against a large panel of pathogens infecting mucosae, using

both *in vitro* models (21, 37, 38) and more demanding *in vivo* models of infection (39–41).

Plasma is an appropriate source of rare proteins with a tremendous field of application in human medicine. Clotting factors, albumin, IgG Abs are already used compounds with established health benefits. In patients with primary or secondary immunodeficiency, replacement therapy with IgG effectively prevents severe systemic infection. However, despite appropriate IgG supplementation, frequently occurring chronic infection/inflammation of the respiratory and gastrointestinal mucosae has been linked to low serum IgA levels (42–44). The inherent structural features of SIgA would make it a logical agent to fill this therapeutic gap in combination with standard therapy (*e.g.* antibiotics, anti-virals, and IgG). Importantly, the combination of two crucial functional properties, namely antigen recognition via Fab (similar to IgG), and anti-inflammatory effector function via Fc is an additional argument to consider IgA in prevention/treatment of mucosal infection and particularly of inflammation associated with chronic infection (45–47). Starting from 1 liter of plasma, current purification procedures allow to recover 40–50 mg of J chain-containing pIgA and up to 100 mg of IgM. Production from 10,000 liters as this is classically performed will yield Abs in sufficient amounts to consider clinical applications. In conclusion, passive topical administration of polyreactive SIgA as well as SIgM may represent a valuable therapeutic approach to control infection before the immune system of the host can take over.

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REFERENCES

1. Corthésy, B. (2010) Role of secretory immunoglobulin A and secretory component in the protection of mucosal surfaces. *Future Microbiol.* **5,** 817–829
2. Brandtzaeg, P. (2009) Mucosal immunity: induction, dissemination, and effector functions. *Scand. J. Immunol.* **70,** 505–515
3. Macpherson, A. J., McCoy, K. D., Johansen, F. E., and Brandtzaeg, P. (2008) The immune geography of IgA induction and function. *Mucosal Immunol.* **1,** 11–22
4. Kaetzel, C. S. (2005) The polymeric immunoglobulin receptor: bridging innate and adaptive immune responses at mucosal surfaces. *Immunol. Rev.* **206,** 83–99
5. Woof, J. M., and Mestecky, J. (2005) Mucosal immunoglobulins. *Immunol. Rev.* **206,** 64–82
6. Woof, J. M., and Russell, M. W. (2011) Structure and function relationships in IgA. *Mucosal Immunol.* **4,** 590–597
7. Brandtzaeg, P. (2003) Mucosal immunity: integration between mother and the breast-fed infant. *Vaccine* **21,** 3382–3388
8. Wijburg, O. L., Uren, T. K., Simpfendorfer, K., Johansen, F. E., Brandtzaeg, P., and Strugnell, R. A. (2006) Innate secretory antibodies protect against natural *Salmonella typhimurium* infection. *J. Exp. Med.* **203,** 21–26
9. Corthésy, B. (2003) Recombinant secretory immunoglobulin A in passive immunotherapy: linking immunology and biotechnology. *Curr. Pharm. Biotechnol.* **4,** 51–67
10. Corthésy, B. (2002) Recombinant immunoglobulin A: powerful tools for fundamental and applied research. *Trends Biotechnol.* **20,** 65–71
11. Cramer, M., Frei, R., Sebald, A., Mazzoletti, P., and Maeder, W. (2009) Stability over 36 months of a new liquid 10% polyclonal immunoglobulin product (IgPro10, Privigen) stabilized with L-proline. *Vox Sang.* **96,** 219–225
12. Phalipon, A., Cardona, A., Kraehenbuhl, J. P., Edelman, L., Sansonetti, P. J., and Corthésy, B. (2002) Secretory component: a new role in secretory IgA-mediated immune exclusion *in vivo*. *Immunity* **17,** 107–115
13. Mathias, A., and Corthésy, B. (2011) Recognition of gram-positive intestinal bacteria by hybridoma- and colostrum-derived secretory immunoglobulin A is mediated by carbohydrates. *J. Biol. Chem.* **286,** 17239–17247
14. Crottet, P., Cottet, S., and Corthésy, B. (1999) Expression, purification and biochemical characterization of recombinant murine secretory component: a novel tool in mucosal immunology. *Biochem. J.* **341,** 299–306
15. Lüllau, E., Heyse, S., Vogel, H., Marison, I., von Stockar, U., Kraehenbuhl, J. P., and Corthésy, B. (1996) Antigen binding properties of purified immunoglobulin A and reconstituted secretory immunoglobulin A antibodies. *J. Biol. Chem.* **271,** 16300–16309
16. Hendrickson, B. A., Conner, D. A., Ladd, D. J., Kendall, D., Casanova, J. E., Corthesy, B., Max, E. E., Neutra, M. R., Seidman, C. E., and Seidman, J. G. (1995) Altered hepatic transport of immunoglobulin A in mice lacking the J chain. *J. Exp. Med.* **182,** 1905–1911
17. Rindisbacher, L., Cottet, S., Wittek, R., Kraehenbuhl, J. P., and Corthésy, B. (1995) Production of human secretory component with dimeric IgA binding capacity using viral expression systems. *J. Biol. Chem.* **270,** 14220–14228
18. Favre, L. I., Spertini, F., and Corthésy, B. (2003) Simplified procedure to recover recombinant antigenized secretory IgA to be used as a vaccine vector. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* **786,** 143–151
19. Crottet, P., and Corthésy, B. (1998) Secretory component delays the conversion of secretory IgA into antigen-binding competent F(ab′)2: a possible implication for mucosal defense. *J. Immunol.* **161,** 5445–5453
20. Berdoz, J., Blanc, C. T., Reinhardt, M., Kraehenbuhl, J. P., and Corthésy, B. (1999) *In vitro* comparison of the antigen-binding and stability properties of the various molecular forms of IgA antibodies assembled and produced in CHO cells. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 3029–3034
21. Cottet, S., Corthésy-Theulaz, I., Spertini, F., and Corthésy, B. (2002) Microaerophilic conditions permit to mimic *in vitro* events occurring during *in vivo Helicobacter pylori* infection and to identify Rho/Ras-associated proteins in cellular signaling. *J. Biol. Chem.* **277,** 33978–33986
22. Rathman, M., Jouirhi, N., Allaoui, A., Sansonetti, P., Parsot, C., and Tran Van Nhieu, G. (2000) The development of a FACS-based strategy for the isolation of *Shigella flexneri* mutants that are deficient in intercellular spread. *Mol. Microbiol.* **35,** 974–990
23. Mestecky, J., and McGhee, J. R. (1987) Immunoglobulin A (IgA): molecular and cellular interactions involved in IgA biosynthesis and immune response. *Adv. Immunol.* **40,** 153–245
24. Johansen, F. E., Braathen, R., and Brandtzaeg, P. (2000) Role of J chain in secretory immunoglobulin formation. *Scand. J. Immunol.* **52,** 240–248
25. Perrier, C., Sprenger, N., and Corthésy, B. (2006) Glycans on secretory component participate in innate protection against mucosal pathogens. *J. Biol. Chem.* **281,** 14280–14287
26. Stubbe, H., Berdoz, J., Kraehenbuhl, J. P., and Corthésy, B. (2000) Polymeric IgA is superior to monomeric IgA and IgG carrying the same variable domain in preventing *Clostridium difficile* toxin A damaging of T84 monolayers. *J. Immunol.* **164,** 1952–1960
27. Almogren, A., Senior, B. W., and Kerr, M. A. (2007) A comparison of the binding of secretory component to immunoglobulin A (IgA) in human colostral S-IgA1 and S-IgA2. *Immunology* **120,** 273–280
28. Brewer, J. W., and Corley, R. B. (1997) Late events in assembly determine the polymeric structure and biological activity of secretory IgM. *Mol. Immunol.* **34,** 323–331
29. Kaetzel, C. S. (2001) Polymeric Ig receptor: defender of the fort or Trojan horse? *Curr. Biol.* **11,** R35–38
30. Corthésy, B., Benureau, Y., Perrier, C., Fourgeux, C., Parez, N., Greenberg, H., and Schwartz-Cornil, I. (2006) Rotavirus anti-VP6 secretory immunoglobulin A contributes to protection via intracellular neutralization but not via immune exclusion. *J. Virol.* **80,** 10692–10699
31. Fernandez, M. I., Pedron, T., Tournebize, R., Olivo-Marin, J. C., Sansonetti, P. J., and Phalipon, A. (2003) Anti-inflammatory role for intracellular dimeric immunoglobulin a by neutralization of lipopolysaccharide in epithelial cells. *Immunity* **18,** 739–749
32. Renegar, K. B., and Small, P. A., Jr. (1991) Passive transfer of local immunity to influenza virus infection by IgA antibody. *J. Immunol.* **146,**

1972–1978
33. Carbonare, C. B., Carbonare, S. B., and Carneiro-Sampaio, M. M. (2005) Secretory immunoglobulin A obtained from pooled human colostrum and milk for oral passive immunization. *Pediatr. Allergy Immunol.* **16,** 574–581
34. Parr, E. L., Bozzola, J. J., and Parr, M. B. (1995) Purification and measurement of secretory IgA in mouse milk. *J. Immunol. Methods.* **180,** 147–157
35. Norderhaug, I. N., Johansen, F. E., Krajci, P., and Brandtzaeg, P. (1999) Domain deletions in the human polymeric Ig receptor disclose differences between its dimeric IgA and pentameric IgM interaction. *Eur. J. Immunol.* **29,** 3401–3409
36. Arranz, E., O'Mahony, S., Barton, J. R., and Ferguson, A. (1992) Immunosenescence and mucosal immunity: significant effects of old age on secretory IgA concentrations and intraepithelial lymphocyte counts. *Gut* **33,** 882–886
37. Hachani, A., Biskri, L., Rossi, G., Marty, A., Ménard, R., Sansonetti, P., Parsot, C., Van Nhieu, G. T., Bernardini, M. L., and Allaoui, A. (2008) IpgB1 and IpgB2, two homologous effectors secreted via the Mxi-Spa type III secretion apparatus, cooperate to mediate polarized cell invasion and inflammatory potential of *Shigella flexenri*. *Microbes Infect.* **10,** 260–268
38. Strauman, M. C., Harper, J. M., Harrington, S. M., Boll, E. J., and Nataro, J. P. (2010) Enteroaggregative *Escherichia coli* disrupts epithelial cell tight junctions. *Infect. Immun.* **78,** 4958–4964
39. Corthésy, B., Boris, S., Isler, P., Grangette, C., and Mercenier, A. (2005) Oral immunization of mice with lactic acid bacteria producing *Helicobacter pylori* urease B subunit partially protects against challenge with *Helicobacter felis*. *J. Infect. Dis.* **192,** 1441–1449
40. Boullier, S., Tanguy, M., Kadaoui, K. A., Caubet, C., Sansonetti, P., Corthésy, B., and Phalipon, A. (2009) Secretory IgA-mediated neutralization of *Shigella flexneri* prevents intestinal tissue destruction by down-regulating inflammatory circuits. *J. Immunol.* **183,** 5879–5885
41. Fournier, B., Williams, I. R., Gewirtz, A. T., and Neish, A. S. (2009) Toll-like receptor 5-dependent regulation of inflammation in systemic *Salmonella enterica* serovar typhimurium infection. *Infect. Immun.* **77,** 4121–4129
42. Gregersen, S., Aaløkken, T. M., Mynarek, G., Kongerud, J., Aukrust, P., Frøland, S. S., and Johansen, B. (2009) High resolution computed tomography and pulmonary function in common variable immunodeficiency. *Respir. Med.* **103,** 873–880
43. Oksenhendler, E., Gérard, L., Fieschi, C., Malphettes, M., Mouillot, G., Jaussaud, R., Viallard, J. F., Gardembas, M., Galicier, L., Schleinitz, N., Suarez, F., Soulas-Sprauel, P., Hachulla, E., Jaccard, A., Gardeur, A., Théodorou, I., Rabian, C., Debré, P., and DEFI Study Group (2008) Infections in 252 patients with common variable immunodeficiency. *Clin. Infect. Dis.* **46,** 1547–1554
44. Quinti, I., Soresina, A., Guerra, A., Rondelli, R., Spadaro, G., Agostini, C., Milito, C., Trombetta, A. C., Visentini, M., Martini, H., Plebani, A., and Fiorilli, M. (2011) Effectiveness of immunoglobulin replacement therapy on clinical outcome in patients with primary antibody deficiencies: results from a multicenter prospective cohort study. *J. Clin. Immunol.* **31,** 315–322
45. Eckmann, L., and Neish, A. S. (2011) NF-κB and mucosal homeostasis. *Curr. Top. Microbiol. Immunol.* **349,** 145–158
46. Mantis, N. J., Rol, N., and Corthésy, B. (2011) Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal. Immunol.* **4,** 603–611
47. Honda, K., and Littman, D. R. (2012) The microbiome in infectious disease and inflammation. *Annu. Rev. Immunol.* **30,** 759–795

Part II: Reconstituted human polyspecific plasma-derived secretory-like IgM and IgA regulate homeostasis of epithelial cells infected with an enteropathogen

Overview of this part

We previously established that human plasma can be used as a source of polyreactive pIgA and IgM to generate secretory-like IgA and IgM Abs. In addition, we demonstrated that pIgA and secretory-like IgA delayed damage to intestinal epithelial polarized Caco-2 cell monolayers induced by a pathogenic strain of *S. flexneri*. Using the same experimental setting, we further dissected the mechanisms of protection of human plasma IgA and secretory-like IgA and examined the functionality of human plasma IgM and secretory-like IgM. We found that human plasma IgM and secretory-like IgM were superior to plasma pIgA and secretory-like IgA in maintaining transepithelial electrical resistance (TER) and in preventing damage of cell monolayers induced by *S. flexneri* infection. We demonstrated that all polyreactive Abs were able to interact with *S. flexneri* but bacterial aggregation was only observed with pIgA and secretory-like IgA, and further amplified with IgM and secretory-like IgM. We observed that all Ab isotypes and molecular forms tested reduced bacterial internalization into Caco-2 cell monolayers. However, only polymeric and secretory-like Abs diminished secretion of pro-inflammatory mediators by cell monolayers. Moreover, we demonstrated that secretory-like IgA and IgM led to a diminution in secretion of *S. flexneri* "virulence" factors IpaB and IpaC. The sum of these data suggests a dual mechanism of action of these Abs combining a direct action on virulence of bacteria and protection of the target epithelium.

Experimental procedures, results, discussion

The results obtained in this part are compiled in the manuscript to be submitted entitled:

Reconstituted human polyspecific plasma-derived secretory-like IgM and IgA regulate homeostasis of epithelial cells infected with an enteropathogen. Longet S (see author contribution), Vonarburg C, Loetscher M, Miescher SM, Zuercher AW, Corthésy B.

Author contribution

I have performed all experiments and analyses related to the study. I have substantially contributed to the writing of the paper under the supervision of Blaise CORTHESY, thesis director.

Reconstituted human polyspecific plasma-derived secretory-like IgM and IgA regulate homeostasis of epithelial cells infected with an enteropathogen

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*Running title: *Homeostatic properties of secretory-like plasma IgA and IgM*

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Keywords: Secretory IgA; secretory IgM; human plasma; epithelial cell

Abstract

Intravenous administration of polyclonal and monoclonal antibodies has proven a clinically valid approach in the treatment or at least relief, of many acute and chronic pathologies including infection, immunodeficiency, and a broad range of autoimmune conditions. IgG immunoglobulins isolated from plasma or from recombinant origin finds the largest application, with occasional use of IgM in therapeutic protocols. We have lately established that secretory-like IgA and IgM can be produced upon association of plasma-derived polymeric IgA and IgM with recombinant secretory component (Longet et al., 2013). As a first step toward future mucosal administration, we sought to unravel the mechanisms whereby these secretory Igs achieve their function of protection toward epithelial cells at the interface between the environment and the inside of the body. By using polarized epithelial Caco-2 cell monolayers and *Shigella flexneri* as a model enteropathogen, we found that polyspecific plasma-derived SIgA and SIgM fulfill many protection function including dose-dependent recognition of the antigen via formation of aggregated immune complexes, maintenance of the epithelial cell integrity, reduction of bacterial infectivity, and inhibition of pro-inflammatory mediators by epithelial cell. In this in vitro model devoid of other cellular or molecular interfering partners, bacterial neutralization by IgM and secretory IgM resulted in better protection than secretory IgA. The sum of these data provides evidence that mucosal passive delivery of antibody preparations will have to rely on both secretory-like IgA and IgM, which together play a crucial role in preserving multi-layers of epithelial cell integrity.

Introduction

Mucosal surfaces constantly exposed to a large variety of pathogens are protected by multi-layer defense mechanisms. Among these, specific humoral mucosal immunity is dominated by secretory antibodies (Abs): secretory immunoglobulin A (SIgA) and secretory immunoglobulin M (SIgM). SIgM results from the transport across the epithelium of J chain-containing pentameric IgM by the polymeric immunoglobulin receptor and exerts a role of neutralizing antibody (1) (2). As the most conserved Ab among vertebrate species, the importance of IgM has been appreciated for several decades. It combines the properties of existing at the beginning of the immune development (3) and is known to be crucial in the primary mucosal immune response (4). Moreover, IgM is able to compensate for the lack of IgA in IgA-deficient individuals (5).

In vitro and *in vivo* studies have established the potential of specific, antigen-induced IgM in systemic neutralization of viruses (2) (6) (7), bacteria (8) (9) (10), fungi (11) and parasites (12) (13) (14). Important advances have especially come from the use of IgM-deficient mice (15), which exhibited high sensitivity to bacterial and viral infections (16), a condition that could be partly controlled upon administration of normal mouse immune serum (6).

Immunotherapy based on the passive administration of human plasma-derived IgG has been used for three decades in clinical applications with improvement of a large panel of disease conditions like immunodeficiencies, infections or autoimmune diseases (17) (18). Pre-clinical and clinical studies have underscored the efficacy against various infectious agents of polyclonal IgM-enriched preparations administered by the *systemic*

route (19) (20) (21) (22) (23). Similar to SIgA, SIgM can be seen as a valid candidate immunoglobulin for mucosal application, given its ability to bind antigens with strong avidity, its potential to ensure long-term protection (24), as well as its resistance to proteases (25). We have recently demonstrated that human plasma can serve as a source of polyreactive, polymeric IgA (pIgA) and IgM to generate secretory-like IgA and IgM Abs, the natural molecular form found in secretions. We found that plasma-derived purified pIgA and IgM can associate recombinant secretory component (SC) with a 1:1 stoichiometry and this association delayed degradation of pIgA or IgM toward intestinal washes containing proteases. In addition to these essential biochemical features, we showed that pIgA and secretory-like IgA delayed damages to epithelial polarized Caco-2 cell monolayers induced by a virulent strain of enteropathogenic *Shigella flexneri* (*S. flexneri*) (25). However, how the plasma-derived Ab operates to block the bacterium and contributes to epithelial homeostasis was not addressed in this study.

To provide answers to these open questions, we sought to further dissect the mechanisms of protection conferred by plasma-derived pIgA and secretory-like IgA, and in addition to evaluate the functionality of human plasma IgM and secretory-like IgM in the same experimental setting. We found a superior ability of IgM or secretory-like IgM compared to pIgA or secretory-like IgA to maintain transepithelial electrical resistance (TER) and to prevent damage of cell monolayers resulting from *S. flexneri* infection. Bacterial aggregates formed with both plasma pIgA and secretory-like IgA, a phenomenon amplified upon association with IgM and secretory-like IgM, consistent with the capacity of all polyreactive Ab molecules to recognize *S. flexneri*. Diminished intracellular bacterial load varies as a function of the Ab isotype, and resulted in

differential production of pro-inflammatory mediators by the Caco-2 cell monolayers. In addition, incubation of secretory-like IgA and IgM resulted in reduced secretion of *S. flexneri* virulence factors IpaB and IpaC, overall suggesting a dual mode of action of the Abs combining disabling of the bacteria and shielding of the target epithelium.

Materials and methods

Preparation of human plasma IgA-, IgM- and IgG-enriched fractions - IgA and IgM were purified from process intermediates of immunoglobulins manufactured from human plasma (26) by affinity chromatography using CaptureSelect Human IgA and CaptureSelect Human IgM resins (Bioaffinity Company BAC). Starting materials used was a chromatography side fraction consisting of the strip fraction from an ion-exchange chromatography column used in the large scale manufacture of IgG from human plasma. The different starting materials were diluted in PBS to a target protein (IgA or IgM) concentration of approximately 1 mg/ml and then loaded onto a CaptureSelect Human IgA or IgM column pre-equilibrated with PBS, without exceeding the IgA- or IgM-binding capacity of the column. After loading the column was washed with PBS, and IgA or IgM was eluted with glycine buffer at pH 3.0. The eluate was adjusted with 0.5 M Tris-base (pH 8.0) to pH 4.5 and concentrated up to 16 mg/ml protein. Human plasma IgG preparations (IgPro10, Privigen) were prepared as described (26).

Separation of plasma-derived pIgA and mIgA and purification of plasma-derived IgM - IgA-enriched preparations containing a mixture of monomeric (mIgA) and pIgA were diluted in PBS to a final volume of 10 ml suitable for injection onto the ÄKTA*prime* chromatography system (GE Healthcare). The flow rate was set at 1 ml/min with PBS as mobile phase for all runs. Separation of the two molecular forms of IgA was performed on two serially coupled 1-meter long columns filled with Sephacryl S-300 HR beads (27). The IgA content of 3.5-ml fractions was verified by immunodetection using polyclonal rabbit anti-human IgA/HRP (1/3000, Dako) and pools of mIgA and pIgA were obtained. IgM-enriched preparations run under identical conditions yielded a single fully-

excluded peak. The IgM content of 3.5-ml fractions was verified by immunodetection using polyclonal rabbit anti-human IgM/HRP (1/3000, Dako). Concentration was performed using the Labscale system (Millipore) connected to a 100-kDa cut-off cartridge, and stored at 4 °C until further use.

In vitro association of polymeric Ig and SC - Recombinant hSC (hSCrec) was produced from a CHO clone stably transfected with an expression cassette coding for the protein (28). Plasma-derived SIgA and SIgM molecules were obtained by combining *in vitro* 10 μg of purified pIgA or IgM molecules with 2.5 μg or 1.5 μg of hSCrec, respectively. Mouse SIgAC5 specific for *S. flexneri* LPS serotype 5a was obtained by combining *in vitro* 10 μg of purified pIgAC5 molecules with 2.5 μg of mSC. Association and characterization of SIgA and SIgM Abs were performed in PBS for 30 min at room temperature (RT) as previously described (25).

Caco-2 cell culture and growth as polarized monolayer - The human colonic adenocarcinoma epithelial Caco-2 cells (American Type Tissue Collection) were grown in complete DMEM consisting of DMEM-Glutamax (Invitrogen) supplemented with 10% FCS (Sigma), 10 mM HEPES (Invitrogen), 1% non essential amino acids (Invitrogen), 1% Sodium Pyruvate (Invitrogen), 1% L-glutamine (Sigma), 1% penicillin/streptomycin (Sigma) and 0.1% transferrin (Invitrogen), and used between passages 32 and 40. Cells cultivated up to 80% confluency were seeded on polyester Snapwell filters (diameter, 12 mm; pore size, 0.4 μm; Corning Costar) at a density of 0.8 x 10^5 cells/cm^2. At week 3, the Caco-2 cell monolayer integrity was checked by measuring the TER using the Millicell-ERS device (Millipore) (29). TER values of well differentiated monolayers were in the range of 400-500 ohms per cm^2.

Bacterial strain and culture conditions - Bacteria used were the virulent strain of serotype 5a LPS *S. flexneri* M90T constitutively expressing green fluorescent protein (GFP) (30). Bacteria from frozen stock were grown in Luria-Bertani (LB) agar plate containing 0.1‰ Congo Red (Applichem) and 50 µg/ml ampicillin (Sigma-Aldrich), for 30 h at 37 °C. Three red colonies were amplified in 10 ml LB liquid broth supplemented with 50 µg/ml ampicillin for 16 h at 37 °C and 200 rpm. The culture was centrifuged at 2'000 x *g* for 5 min, resuspended in PBS, diluted 1/10 in 10 ml LB liquid/ampicillin and then cultured for 2 h at 37 °C with shaking (200 rpm). Finally, bacteria in the exponential phase were washed twice in PBS by centrifugation at 2'000 x *g* for 5 min and resuspended in PBS. Assessment of colony forming unit (cfu)/ml was carried out by measurement of the optical density (OD) at 600 nm with the knowledge that 1 OD unit at 600 nm corresponds to 5×10^8 cfu/ml.

Incubation of bacteria with different Ab preparations - 2×10^7 bacteria were mixed with 0.049 µM of SIgAC5 specific for *S. flexneri* LPS serotype 5a or with human plasma-derived pIgA (0.61 µM), reconstituted SIgA (0.61 µM), mIgA (0.61 µM), IgM (0.61 µM), reconstituted SIgM (0.61 µM) or IgG (0.61 µM). All mixtures were prepared in a final volume of 500 µl of plain DMEM (P-DMEM: DMEM complemented with 10 mM HEPES, 1% non essential amino acids, 1% sodium pyruvate, 1% L-glutamine and 0.1% transferrin). The mixtures were incubated for 1 h at RT under gentle agitation.

Protection assay - One h before the use of polarized Caco-2 cell monolayers, C-DMEM was replaced by P-DMEM in both the apical and basolateral compartments. Polarized Caco-2 cell monolayers were infected apically with *S. flexneri* serotype 5a alone or in combination with the Ab preparations. Exposure of Caco-2 cells to antigens

or the various immune complexes was performed overnight (O/N) and pathogen-induced damages were tracked by measuring TER decrease timewise.

Counting of associated bacteria to cell monolayers – To numerate internalized bacteria, cells on Snapwell filters were washed in PBS, and incubated for 30 min with 50 µg/ml gentamicin. Following incubation in 500 µl of cold lysis buffer [10 mM Tris-HCl (pH 7), 0.2 % Nonidet P-40, 50 mM NaCl, 2 mM EDTA (pH 8)] for 5 min on ice and lysed by up-and-down pipetting. *S. flexneri* present in cell lysates were numerated from serial dilutions seeded onto LB-agar plates.

Laser-scanning confocal microscopy (LSCM) observation of Caco-2 cell monolayers - To examine the integrity of Caco-2 cell monolayers, Snapwells were washed twice with PBS, prior to fixation O/N with 5 ml of 4% paraformaldehyde at 4 °C. After washing, filters were permeabilized and non-specific binding sites were blocked using PBS containing 5% FCS and 0.2% Triton X-100 for 30 min at RT. All Abs were diluted in PBS containing 0.05% of Tween 20 (PBS-T). Filters were incubated with phalloidin associated to Fluoprobes 547H (1/200, Interchim) for 90 min at RT and washed in PBS. To visualize cells, filters were finally incubated with 200 ng/ml of 4',6'-diamidino-2-phenylindole (DAPI; Invitrogen) in PBS for 30 min. Filters were cut out of their holders, and mounted in Vectashield solution (Vector Laboratories) for observation using Zeiss LSM 710 Meta confocal microscope (Carl Zeiss, Germany) equipped with a 40x objective. Snapshots of x-plan slices were performed with the Zeiss ZEN 2009 light software.

Quantification of the infected areas and the number of infection foci - Observation of whole filters was carried out with the 10x objective using Zeiss ZEN 2009 light software.

The sum of infected areas and the number of infection foci were determined using the particle analysis tool of Image J software applied onto the channel associated with the bacteria.

LSCM observations of immune complexes - The formation of immune complexes was verified after incubation with biotinylated mouse anti-human IgA1/IgA2 (1/10, BD) for 30 min at RT under gentle agitation, followed by cyanine 5-conjugated Streptavidin (1/400, GE HealthCare) for 30 min at RT under gentle agitation. Three washes with PBS were performed between each step and all Abs were diluted in PBS/5% FCS. Labeled immune complexes as such were laid onto glass slides (Thermo Scientific), mounted and immediately visualized using a Zeiss LSM 710 Meta confocal microscope (Carl Zeiss, Germany) equipped with a 40x objectives. Images were processed with the Zeiss ZEN 2009 light software.

Enzyme-linked immunosorbent assay (ELISA) - Human CXCL8, TNF-α, and CCL3 in the basolateral compartment of polarized Caco-2 cell monolayers infected by *S. flexneri* alone or combined to Abs were measured by ELISA using commercial kits (BD Biosciences and R&D Systems, respectively). To examine the binding capacity of human plasma IgA/IgM to bacteria, 96-well plates (MaxiSorp, Nunc) were coated with 4 x 10^7 cfu/well of *S. flexneri* serotype 5a in PBS O/N at 4°C. After three washes in PBS-T, wells were blocked with PBS-T containing 1% bovine serum albumin (BSA, Fluka) for 1 h at RT. Serial dilutions of human plasma IgA, SIgA, IgM, SIgM, IgG or mouse SIgAC5 (from 0.61 µM) were incubated in wells for 2 h at RT, washed with PBS-T, and detection was performed by incubation with isotype-specific Abs: mouse anti-human IgA1/IgA2 biotinylated IgG (BD, 1/1000), goat anti-human mu chain biotinylated IgG

(KPL, 1/1’000), goat anti-human gamma chain biotinylated IgG (Sigma, 1/1’000) or goat anti-mouse alpha chain biotinylated IgG (KPL, 1/1’000 dilution) for 2 h at RT followed by Extravidin-HRP (Sigma, 1/5’000 dilution) for 1 h at RT. All samples and Ab dilutions were performed in PBS-T/0.1% BSA. Finally, detection was performed with citrate/phosphate solution (44.4 mM citric acid, 103 nM Na_2HPO_4, pH 5.0) containing 1 mg/ml O-phenylenediamine (Sigma) and 0.01% H_2O_2. The reactions were stopped with 1 M H_2SO_4. Absorbance was read at 490 nm with 630 nm as reference.

Measurement of virulence factors IpaB and IpaC secreted by Shigella flexneri - To examine the impact of Abs on the expression of virulence factors, the immune complexes bacteria-Abs were formed as previously described, and left for another h at RT. To induce T3SS-mediated secretion of invasion plasmid antigens (Ipas), bacteria and immune complexes were exposed to 6 µg/ml Congo red for 10 min at 37 °C (31) (32). The supernatants were recovered by centrifugation and kept at -20 °C prior to use. The presence of secreted IpaB and IpaC in the supernatant was analyzed by immunodetection using 1/1’000 dilutions of specific mouse monoclonal Abs (kindly provided by Dr. Edwin Oaks, WRAIR, Silver Spring, MD), followed by HRP-conjugated rabbit anti-mouse IgG (Sigma, 1/3’000).

Statistical analysis - Results were expressed as mean ± standard error of the mean. Student’s *t*-test analysis was performed using GraphPad 6 Prism software. Differences were considered as significant when p values < 0.05.

Results

Human plasma pIgA and SIgA Abs induce bacterial agglutination leading to a decrease of the bacterial load in Caco-2 cell monolayers and of cell-produced pro-inflammatory mediators

We previously demonstrated that human plasma-derived pIgA and reconstituted SIgA, but not plasma mIgA, allowed a significant maintenance of Caco-2 cell intestinal epithelial monolayer integrity after O/N infection with *S. flexneri*, as reflected by 1) weak reduction of TER; 2) preservation of the tight junction network; 3) limited detachment of filter-bound Caco-2 cells (25). However, the underlying mechanisms explaining protection were not tackled. To better define the mode of action of human plasma pIgA and SIgA, we first got interested in the nature of the interaction between the bacteria and the Abs. The binding capacity of the various molecular forms of plasma IgA to *S. flexneri* was compared by ELISA. All molecular forms of IgA demonstrated a concentration-dependent ability to recognize the bacteria. At identical concentrations, the signal for pIgA or SIgA was 3 to 4-fold more important compared to the signal observed with mIgA (Fig. 1A), emphasizing the avidity effect associated with the polymeric structure of the Ab. To get insight into the nature of the interaction, immune complexes between Cy5-labeled Abs and GFP-expressing bacteria were formed and directly visualized by LSCM. Specific SIgAC5 and plasma-derived IgG were assessed for comparison. All molecular forms bound to *S. flexneri*, yet bacterial aggregates of increasing size formed upon association with human plasma pIgA and SIgA, suggesting that only tetravalent IgA's molecular structures were prone to trigger the assembly of

complex lattices (Fig. 1B). Interestingly, the large aggregate pattern resembles that formed with *S. flexneri* LPS-specific SIgAC5 (33).

As differences in Caco-2 cell monolayer integrity (25) and pattern of immune complexes were identified (Fig. 1B) as a function of the various molecular forms, we examined whether this correlated with infection by *S. flexneri*. We found that the number of internalized bacteria by cell monolayers was on an average 3 times less when either of the polyreactive Abs was present on the top of *S. flexneri*, while specific SIgAC5 mAb offered the best blocking to bacterial entry (Fig. 2A). In the same experimental setting, we then studied the effect of the molecular forms of plasma IgA on the inflammatory response of infected cell monolayers. In the presence of pIgA or SIgA, basolateral secretion of TNF-α, CCL3 and CXCL8 fell to less than half the level of production measured upon infection with *S. flexneri* alone (Fig. 2B). This drop in cytokine/chemokine production was in the same range as that obtained with specific SIgAC5, whereas mIgA and (monomeric) IgG had little effect on CXCL8 secretion only (Fig. 2B). We conclude that the various molecular forms of plasma IgA and plasma IgG directly bind to bacteria, yet with various consequences on infection or responsiveness of target epithelial cells that appears to individually depend on the size of formed immune complexes. The implication of such data on protection against invading enteropathogens is discussed in the final section of the paper.

Human plasma IgM and SIgM Abs efficiently prevent damages to epithelial Caco-2 cell monolayers infected by S. flexneri

As the avidity properties of polyspecific pIgA/SIgA are crucial to protect Caco-2 cell monolayers with the best efficacy against infection by *S. flexneri*, we sought to

determine whether polyreactive plasma-derived pentameric IgM of even higher avidity would achieve improved performance. To this aim, human plasma-derived IgM and reconstituted SIgM at the same molar concentration as SIgA serving as a reference control were combined with *S. flexneri*, and incubated O/N with Caco-2 cell monolayers. The resulting integrity of cell monolayers was assessed by TER value measurement, cell morphology, as well as through the number of, and total surface of, infected foci on whole filters. In contrast to *S. flexneri* alone, TER was maintained when the bacteria were mixed with IgM or SIgM, close to values measured with SIgA (Fig. 3A). Representative snapshots of transversal sections obtained along the x-axis of monolayers showed that the infected areas deprived of cells were systematically smaller after incubation with immune complexes comprising IgM and SIgM than with those based on association with SIgA, or with *S. flexneri* alone (Fig. 3B). The same hierarchy in maintenance of the integrity of monolayers was visualized upon staining of the preserved, well-organized actin network (Fig. 3B). When compared with bacteria alone, complexes with plasma SIgA diminished the total surface of infection foci 4 times, while slightly reducing their number (Fig. 3C and D). Plasma IgM and SIgM showed a 10-fold and 6-fold drop for these two parameters, respectively, reflecting low damages inflicted to the monolayer (Figure 3C and D). We conclude that both IgM and SIgM prevent destruction of Caco-2 cell monolayers exposed to infectious *S. flexneri* to a degree overpassing SIgA.

Human plasma IgM and SIgM Abs induce bacterial agglutination reducing interactions between bacteria and Caco-2 cell monolayers

Data in Longet et al. (2013) (25) and Fig. 1 indicate that neutralization by SIgA through bacterial aggregation provides the most comprehensive protection of Caco-2 cell monolayers based on the several parameters assayed. Together with the positive impact both IgM and SIgM Abs have on maintaining the organization of the Caco-2 cell monolayer (Fig. 3), we sought to investigate further by which mechanisms the Ab achieves protection. In term of binding, ELISA data demonstrated a similar concentration-dependent capacity of IgM and SIgM to interact with *S. flexneri* (Fig. 4A). LSCM images allowed to visualize large aggregates comprising bacteria and either IgM or SIgM Abs (Fig. 4B), whose size was well above that detected upon incubation with SIgA. Integrity of the epithelial cell monolayer was highly preserved, with occasional and limited actin fiber depolymerization (Fig. 3B).

This prompted us to speculate that such a strong agglutination capacity may result in blocking internalization of *S. flexneri* by Caco-2 cell monolayers. After O/N infection with the bacteria alone, or in complex with IgM, SIgM and SIgA, cells were treated with gentamicin for 30 min, lysed, and the lysate was plated on a selective medium for numeration. In comparison with bacteria alone, a 10-fold reduction in the number of cell-associated bacteria was measured when complexed with either IgM or SIgM (Fig. 5A), whereas a reproducible 4-fold decrease was observed in the presence of SIgA (Fig. 2A and Fig. 5A). These results illustrate a strong ability of IgM and SIgM to neutralize the bacteria via aggregation and explain data in Fig. 3B which show limited binding and spreading of green *S. flexneri*.

Human plasma IgM and SIgM Abs diminish the secretion of pro-inflammatory chemo- and cytokines by Caco-2 cell monolayers

The finding that plasma IgM and SIgM Abs appear superior to SIgA for all parameters tested so far led us to hypothesize that this should hold true when examining the pro-inflammatory responsiveness of Caco-2 cell monolayers exposed to the bacterium alone or in complex with Abs. Basolateral secretion of TNF-α, CCL3 and CXCL8 measured by ELISA after O/N incubation showed that cell monolayers infected by IgM- or SIgM-*S. flexneri* complexes released 4-fold less of TNF-α and CCXL8, as well as 3-fold less of CCL3 than monolayers infected by the bacteria alone (Fig. 5B). SIgA led to a significantly less important diminution of TNF-α and CCL3 secretion than IgM and SIgM. Hence, neutralization of *S. flexneri* by either IgM or SIgM displays the more marked effect on the pro-inflammatory response of polarized Caco-2 cell monolayer of all Abs tested, and importantly, this occurs in the absence of any other cell partner that could have biased the analysis.

Human plasma secretory-like Abs impact on the secretion of virulence factors IpaB and IpaC released by S. flexneri

In addition to its protective properties, the specific anti-*S. flexneri* IgAC5 mAb allowed a transient suppression of the type 3 secretion system (T3SS) of the bacterium, leading to a decrease in secretion of the virulence factor known as invasion plasmid antigen B (IpaB) (32). In order to examine whether polyspecific human plasma-derived IgA and IgM could act via a similar mechanism, we evaluated the secretion of IpaB and IpaC under conditions used to form immune complexes. *S. flexneri* was associated with human plasma pIgA, SIgA, mIgA, IgM, SIgM, IgG and specific IgAC5 mAb for 1 h, or left as such. While bacteria not exposed to Congo red secreted a low basal level of IpaB and no IpaC, exposure to Congo red led to secretion of various levels of IpaB and IpaC

in supernatants as a function of the complexing Ab. Noteworthy, the level of secretion of IpaB by bacteria in complex with human plasma SIgA, and SIgM was decreased (Fig. 6A and C), while IpaC was affected by SIgA, IgM and SIgM (Fig. 6B and C). In comparison with the bacterium alone, the other molecular forms of IgA, IgM, as well as IgG and SIgAC5 did not lead to detectable changes (Fig. 6). This result suggests that the secretory form of plasma-derived IgA and IgM may contribute indirectly to protection of target epithelial surfaces through its impact on bacterial virulence.

Discussion

We previously established that human plasma-derived pIgA and IgM can be assembled into secretory-like Abs (25). Further, we established that pIgA and SIgA, but not monomeric IgA, reduced damages of polarized Caco-2 cell monolayers infected by a virulent strain of *S. flexneri* (25). However, how the various molecular forms of the Ab displayed differential protection was not evaluated at the cellular and molecular level. The current study addresses these issues, and further extends the analysis to plasma-derived IgM and SIgM. We found that pIgA and IgM or the secretory form of the Ab recognized *S. flexneri* to the same extent, and the interaction led to the formation of aggregates with a size dependent on the valence of the Ab. While reduced bacteria internalization into cell monolayer occurred with all Ab isotypes and molecular forms tested, bacteria-induced damages to the monolayer organization was significantly diminished with polymeric and secretory-like Abs only, as identified by TER measurement, cell imaging, quantification of infectious foci and assessment of areas exhibiting monolayer destruction, as well as secretion of pro-inflammatory mediators. Moreover, plasma-derived Abs were found to disable *S. flexneri* in its capacity to produce IpaB and IpaC, two proteins involved in invasion of epithelial cells. Direct comparison with SIgA led to the conclusion that IgM and SIgM Ab molecules prove superior in preserving the integrity and responsiveness to infection of polarized Caco-2 cell monolayers used as a mimic of the gut mucosal epithelium.

Similar in vitro models have previously been used to compare the neutralizing function of IgG and their IgA/SIgA counterpart (33) (34) (35), yet the studies focused on Abs of well-defined monoclonal specificity on one hand, and IgM/SIgM was not evaluated in

parallel on another hand. The availability of polyreactive IgA and IgM Abs with well characterized biochemical properties (25) turned out to be an asset to address unsolved issues as to their mode of action on polarized epithelial cells serving as a model target mimicking mucosal surfaces. In addition, the various Abs could be examined in the absence of any other cellular and molecular partners involved in clearance of *S. flexneri*, allowing to draw straight conclusions when comparing their respective functional characteristics.

Remarkably, for most of the parameters examined, in comparison with the specific protective SIgAC5 mAb, polyreactive pIgA and SIgA displayed similar degrees of protection at a 10-fold higher concentration only. Both IgM and SIgM prove even more potent; this is particularly true for blocking of internalization, maintenance of the polarized Caco-2 cell monolayer integrity, and reduction in the production of cellular pro-inflammatory mediators. This finds a rationale explanation in the observation that apical immune exclusion appears the most potent for multivalent IgM Abs of the highest valence. Strikingly, agglutination mediated by pIgA and SIgA did not translate in different levels of bacteria internalization in comparison with mIgA and IgG. This contrasts with the observation that LPS-specific monomeric IgAC5 and IgGC20 mAbs performs more poorly than their SIgA counterpart recognizing the same epitope (33). This suggests that the polyspecific nature of plasma-derived mIgA and IgG masking both LPS and bacterial adhesins prevents bacterial entry more efficiently. Alternatively, it may block intracellular bacterial proliferation without impacting on epithelial cell responsiveness, such as morphological changes and secretion of pro-inflammatory mediators. An side observation from this work, this reveals that the sensing of antigens

by epithelial cells might differ according to the molecular form of the Abs found in the immune complexes, as for instance intracellular processing pathways (36).

The presence of SC on reconstituted SIgA and SIgM did not modify by any mean the function of the Ab molecule as assessed inhere, with the notable exception of the effect on IpaB and IpaC secretion by *S. flexneri*. However, one has to remember that in the *in vivo* context, the presence of bound SC will be essential to the stability and anchoring of the molecule at mucosal surfaces (37). Furthermore, in addition to interfere with bacterial targeting of epithelial cells via carbohydrates abundantly found on its surface (38), SC associated with pIgA and IgM appears to intervene negatively in the secretion of *S. flexneri* virulence factors crucial for infection of epithelial cells (39) (40). These results suggest the unexpected role of polyclonal SIgA and SIgM mostly in altering bacterial metabolism, which adds to identified mechanisms of protection effective at the level of the mucosal epithelium.

Previous animal studies have demonstrated that administration of polyclonal IgM molecules, especially derived from human plasma, could be beneficial in case of sepsis. Lachmann et al. (2004) (22) showed that IgM-enriched preparations reduced *Klebsiella pneumoniae* infection using a distress syndrome rat model. Stehr and colleagues (23) emphasized the benefits of polyclonal IgM-enriched solution by using a rabbit model of bacteremia (2008). Clinical trials showed that IgM-enriched preparations were able to significantly decrease endotoxin levels in plasma (20) and even reduce mortality (19) during early phase of septic shock. Norbby-Teglund et al. (2000) (41) showed that IgM-enriched preparations were able to inhibit specific streptococcal antigens. However, a limitation in the strict interpretation of these results, IgM-enriched preparations

contained significant amounts of IgG and/or IgA Ab molecules, thus making it difficult to assign the intrinsic role of polyspecific IgM as such. It remains that these in vivo studies relying on intravenous and intraperitoneal administration, together with the demonstration that IgM/SIgM Abs exhibit functional protective functions toward epithelial cells exposed to an enteropathogen, pave the way to consider mucosal passive delivery of the Ab along the gastrointestinal and nasal routes. This would combine protection and low pro-inflammatory responses by the epithelial cells located at the interface between the environment and the inside of the body. Additionally, given the similar potential of SIgA in quenching pro-inflammatory cellular responses, it seems an interesting development to consider topical application of such molecules to compensate for IgA deficiency often associated with autoimmunity (42).

By interfering with early events resulting from epithelial infection by *S. flexneri*, polyreactive SIgA/SIgM given therapeutically may facilitate epithelial healing by inhibiting destructive process occurring in the frame of infection. More generally, due to their broad range of antigen recognition, both reconstituted secretory Abs deserve now to be evaluated in in vivo models of mucosal pathologies such as infectious diseases and inflammation with well-founded chances of success.

Figure legends

Figure 1: Association of human plasma-derived IgA/SIgA with *Shigella flexneri*. A) Binding of equimolar concentrations of pIgA, reconstituted SIgA or mIgA to immoblized *S. flexneri* as determined by ELISA. Successive dilutions of the various molecular forms of IgA were assessed, with the 1/1 ratio corresponding to 0.61 µM of each respective Ab. The panel is representative of two independent experiments performed in duplicates. B) LSCM images of immune complexes of bacteria associated to human plasma-derived pIgA, SIgA, mIgA, IgG or anti-*S. flexneri* LPS-specific SIgAC5 mAb. Bacteria constitutively expressing GFP appear in green. Bound Abs were detected by antisera directed against the α or γ chain, followed by Abs conjugated to fluorophores yielding red signals after image processing. Displayed pictures are representative of one representative field obtained from 15 observations from three independent slides per experiment. Scale bar, 10 µm.

Figure 2: Modulatory effect of various human plasma-derived IgA/SIgA preparations on Caco-2 cells infected by *Shigella flexneri*. A) Internalization of *S. flexneri* alone or associated with SIgAC5 mAb, and with plasma-derived pIgA, reconstituted SIgA, mIgA and IgG to polarized Caco-2 cell monolayers as determined after O/N incubation, with addition of gentamicin for the last 30 min. Data are expressed on a “per filter” basis, and correspond to one representative experiment (n=2) for each tested condition performed in triplicates. B) Basolateral secretion of TNF-α, CXCL8 and CCL3 by polarized Caco-2 cells monolayers after an O/N incubation with *S. flexneri* alone, or associated to plasma-derived pIgA, SIgA, mIgA, IgG, or anti-*Shigella* LPS-specific SIgAC5 mAb. The concentration of pro-inflammatory mediators was determined

by ELISA. Non-infected Caco-2 cell monolayers (No bacteria) serve as control. Data are the pool of three experiments performed in duplicates (n=6). For both panels A and B, significant statistical differences calculated by comparison with the condition *S. flexneri* alone (Sf) are indicated above the columns: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. nd = non-detectable.

Figure 3: Integrity of Caco-2 cell monolayers infected with *Shigella flexneri* alone or in combination with human plasma-derived IgM/SIgM. A) TER of Caco-2 cell monolayers exposed O/N to *S. flexneri* alone or associated to human IgM, reconstituted SIgM, and plasma-derived SIgA (control), as determined at three time-points. The TER values for each condition and each time-point were normalized to the TER values at time 0, and are expressed as percentages. The panel is representative of one individual experiment (out of five) performed in duplicate. Significant statistical differences calculated by comparison with the condition *S. flexneri* alone (Sf) are indicated above the columns: *, $p < 0.05$; **, $p < 0.01$. B) LSCM pictures (snapshot) of transversal sections obtained along the x-axis of Caco-2 cell monolayers exposed O/N to *S. flexneri* alone or in complex with IgM, SIgM and SIgA. Actin fibers are visualized by phalloidin labeling (red), Caco-2 cells are visualized via nuclear staining with DAPI (blue) and bacteria constitutively expressing GFP stain green. Scale bar, 50 µm. For quantitative analysis, the sum of infected areas (C) and the number of infection foci (D) were determined from LSCM pictures of whole filters using Image J software. Significant statistical differences calculated by comparison with the conditions *S. flexneri* alone (Sf) are indicated above the columns: *, $p < 0.05$; **, $p < 0.01$. Data in panels C and D are representative of three independent experiments performed in duplicates.

Figure 4: Association of human plasma-derived IgM/SIgM with *Shigella flexneri*. A) Binding of IgM and SIgM to *S. flexneri* determined by ELISA. Bacteria coated in well plates were incubated with IgM or reconstituted SIgM at decreasing concentrations, with the 1/1 ratio corresponding to 0.61 µM of either Ab. This panel is representative of two experiments performed in duplicates. B) LSCM pictures of immune complexes formed between bacteria and human plasma-derived IgM or SIgM. Bacteria constitutively expressing GFP show in green, while IgM and SIgM detected μ chain-specific and fluorescent Abs appear in red. Displayed pictures are representative of one representative field obtained from 10 observations from two independent slides per experiment. Scale bar, 10 µm.

Figure 5: Modulatory effect of various human plasma-derived IgM/SIgM preparations on Caco-2 cells infected by *Shigella flexneri*. A) Bacteria internalized within Caco-2 cell monolayers determined after O/N infection by *S. flexneri* alone or in complex with human plasma IgM, reconstituted SIgM or SIgA. Bacterial counts were carried out after addition of gentamicin for the last 30 min of incubation. Data are expressed on a “per filter” basis, and correspond to one representative experiment (n=4) for each tested condition performed in duplicates. B) Basolateral secretion of TNF-α, CXCL8 and CCL3 by polarized Caco-2 cells monolayers after an O/N incubation with *S. flexneri* alone, or associated to plasma-derived IgM, SIgM and SIgA. The concentration of pro-inflammatory mediators was determined by ELISA. Non-infected Caco-2 cell monolayers (No bacteria) serve as control. Data are the pool of two experiments performed in triplicates (n=6). For both panels A and B, significant statistical differences calculated by comparison with the condition *S. flexneri* alone (Sf)

are indicated above the columns: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ***, $p < 0.0001$. nd = non-detectable.

Figure 6: Impact of human plasma-derived IgA and IgM preparations on the secretion of virulence factors IpaB and IpaC by *Shigella flexneri*. Immune complexes between bacteria and human plasma-derived pIgA, SIgA, mIgA, IgG, IgM, SIgM or anti-*Shigella* LPS-specific SIgAC5 mAb were formed for 1 h as described in "Materials and Methods" and expression of virulence factor was induced by Congo red. The secretion of IpaB (A) and IpaC (B) was examined by immunoblot analysis using mAbs directed against IpaB and IpaC. The images are representative of one individual experiment performed in duplicates. C) Densitometric analysis of immunoblots depicted in panels A and B exposed for optimal times to avoid saturation of the photographic film. The intensity of the signals reached with *S. flexneri* alone exposed to Congo red was fixed at 100%.

Bibliography

1. Klimovich, V. B. (2011) IgM and its receptors: structural and functional aspects. *Biochemistry (Mosc)*. **76**, 534-549
2. Diamond, M. S., Sitati, E. M., Friend, L. D., Higgs, S., Shrestha, B., and Engle, M. (2003) A critical role for induced IgM in the protection against West Nile virus infection. *J Exp Med*. **198**, 1853-1862
3. Gronwall, C., Vas, J., and Silverman, G. J. (2012) Protective Roles of Natural IgM Antibodies. *Front Immunol*. **3**, 66
4. Croft, N. M., and Hodges, M. (2005) IgM: mucosal response in acute diarrhoeal disease of infants. *Scand J Gastroenterol*. **40**, 965-971
5. Brandtzaeg, P., Karlsson, G., Hansson, G., Petruson, B., Bjorkander, J., and Hanson, L. A. (1987) The clinical condition of IgA-deficient patients is related to the proportion of IgD- and IgM-producing cells in their nasal mucosa. *Clin Exp Immunol*. **67**, 626-636
6. Baumgarth, N., Herman, O. C., Jager, G. C., Brown, L. E., Herzenberg, L. A., and Chen, J. (2000) B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. *J Exp Med*. **192**, 271-280
7. Seiler, P., Kalinke, U., Rulicke, T., Bucher, E. M., Bose, C., Zinkernagel, R. M., and Hengartner, H. (1998) Enhanced virus clearance by early inducible lymphocytic choriomeningitis virus-neutralizing antibodies in immunoglobulin-transgenic mice. *J Virol*. **72**, 2253-2258

8. Boes, M., Prodeus, A. P., Schmidt, T., Carroll, M. C., and Chen, J. (1998) A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection. *J Exp Med*. **188**, 2381-2386

9. Salinas-Carmona, M. C., and Perez-Rivera, I. (2004) Humoral immunity through immunoglobulin M protects mice from an experimental actinomycetoma infection by Nocardia brasiliensis. *Infect Immun*. **72**, 5597-5604

10. Alugupalli, K. R., Gerstein, R. M., Chen, J., Szomolanyi-Tsuda, E., Woodland, R. T., and Leong, J. M. (2003) The resolution of relapsing fever borreliosis requires IgM and is concurrent with expansion of B1b lymphocytes. *J Immunol*. **170**, 3819-3827

11. Subramaniam, K. S., Datta, K., Quintero, E., Manix, C., Marks, M. S., and Pirofski, L. A. (2010) The absence of serum IgM enhances the susceptibility of mice to pulmonary challenge with Cryptococcus neoformans. *J Immunol*. **184**, 5755-5767

12. Couper, K. N., Roberts, C. W., Brombacher, F., Alexander, J., and Johnson, L. L. (2005) Toxoplasma gondii-specific immunoglobulin M limits parasite dissemination by preventing host cell invasion. *Infect Immun*. **73**, 8060-8068

13. Konishi, E., and Nakao, M. (1992) Naturally occurring immunoglobulin M antibodies: enhancement of phagocytic and microbicidal activities of human neutrophils against Toxoplasma gondii. *Parasitology*. **104 (Pt 3)**, 427-432

14. Baral, T. N., De Baetselier, P., Brombacher, F., and Magez, S. (2007) Control of Trypanosoma evansi infection is IgM mediated and does not require a type I inflammatory response. *J Infect Dis*. **195**, 1513-1520

15. Ehrenstein, M. R., O'Keefe, T. L., Davies, S. L., and Neuberger, M. S. (1998) Targeted gene disruption reveals a role for natural secretory IgM in the maturation of the primary immune response. *Proc Natl Acad Sci U S A*. **95**, 10089-10093

16. Boes, M., Esau, C., Fischer, M. B., Schmidt, T., Carroll, M., and Chen, J. (1998) Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. *J Immunol*. **160**, 4776-4787

17. Orange, J. S., Hossny, E. M., Weiler, C. R., Ballow, M., Berger, M., Bonilla, F. A., Buckley, R., Chinen, J., El-Gamal, Y., Mazer, B. D., Nelson, R. P., Jr., Patel, D. D., Secord, E., Sorensen, R. U., Wasserman, R. L., Cunningham-Rundles, C., Primary Immunodeficiency Committee of the American Academy of Allergy, A., and Immunology. (2006) Use of intravenous immunoglobulin in human disease: a review of evidence by members of the Primary Immunodeficiency Committee of the American Academy of Allergy, Asthma and Immunology. *J Allergy Clin Immunol*. **117**, S525-553

18. Durandy, A., Kaveri, S. V., Kuijpers, T. W., Basta, M., Miescher, S., Ravetch, J. V., and Rieben, R. (2009) Intravenous immunoglobulins--understanding properties and mechanisms. *Clin Exp Immunol*. **158 Suppl 1**, 2-13

19. Schedel, I., Dreikhausen, U., Nentwig, B., Hockenschnieder, M., Rauthmann, D., Balikcioglu, S., Coldewey, R., and Deicher, H. (1991) Treatment of gram-negative septic shock with an immunoglobulin preparation: a prospective, randomized clinical trial. *Crit Care Med*. **19**, 1104-1113

20. Behre, G., Schedel, I., Nentwig, B., Wormann, B., Essink, M., and Hiddemann, W. (1992) Endotoxin concentration in neutropenic patients with suspected gram-negative sepsis: correlation with clinical outcome and determination of anti-endotoxin core antibodies during therapy with polyclonal immunoglobulin M-enriched immunoglobulins. *Antimicrob Agents Chemother*. **36**, 2139-2146

21. Norrby-Teglund, A., Haque, K. N., and Hammarstrom, L. (2006) Intravenous polyclonal IgM-enriched immunoglobulin therapy in sepsis: a review of clinical efficacy in relation to microbiological aetiology and severity of sepsis. *J Intern Med*. **260**, 509-516

22. Lachmann, R. A., van Kaam, A. H., Haitsma, J. J., Verbrugge, S. J., Delreu, F., and Lachmann, B. (2004) Immunoglobulin M-enriched intravenous polyclonal immunoglobulins reduce bacteremia following Klebsiella pneumoniae infection in an acute respiratory distress syndrome rat model. *Exp Lung Res*. **30**, 251-260

23. Stehr, S. N., Knels, L., Weissflog, C., Schober, J., Haufe, D., Lupp, A., Koch, T., and Heller, A. R. (2008) Effects of IGM-enriched solution on polymorphonuclear neutrophil function, bacterial clearance, and lung histology in endotoxemia. *Shock*. **29**, 167-172

24. Racine, R., McLaughlin, M., Jones, D. D., Wittmer, S. T., MacNamara, K. C., Woodland, D. L., and Winslow, G. M. (2011) IgM production by bone marrow plasmablasts contributes to long-term protection against intracellular bacterial infection. *J Immunol*. **186**, 1011-1021

25. Longet, S., Miled, S., Lotscher, M., Miescher, S. M., Zuercher, A. W., and Corthésy, B. (2013) Human plasma-derived polymeric IgA and IgM antibodies

associate with secretory component to yield biologically active secretory-like antibodies. *J Biol Chem*. **288**, 4085-4094

26. Cramer, M., Frei, R., Sebald, A., Mazzoletti, P., and Maeder, W. (2009) Stability over 36 months of a new liquid 10% polyclonal immunoglobulin product (IgPro10, Privigen) stabilized with L-proline. *Vox Sang*. **96**, 219-225
27. Favre, L. I., Spertini, F., and Corthésy, B. (2003) Simplified procedure to recover recombinant antigenized secretory IgA to be used as a vaccine vector. *J Chromatogr B Analyt Technol Biomed Life Sci*. **786**, 143-151
28. Phalipon, A., Cardona, A., Kraehenbuhl, J. P., Edelman, L., Sansonetti, P. J., and Corthésy, B. (2002) Secretory component: a new role in secretory IgA-mediated immune exclusion in vivo. *Immunity*. **17**, 107-115
29. Cottet, S., Corthésy-Theulaz, I., Spertini, F., and Corthésy, B. (2002) Microaerophilic conditions permit to mimic in vitro events occurring during in vivo Helicobacter pylori infection and to identify Rho/Ras-associated proteins in cellular signaling. *J Biol Chem*. **277**, 33978-33986
30. Rathman, M., Jouirhi, N., Allaoui, A., Sansonetti, P., Parsot, C., and Tran Van Nhieu, G. (2000) The development of a FACS-based strategy for the isolation of Shigella flexneri mutants that are deficient in intercellular spread. *Mol Microbiol*. **35**, 974-990
31. Bahrani, F. K., Sansonetti, P. J., and Parsot, C. (1997) Secretion of Ipa proteins by Shigella flexneri: inducer molecules and kinetics of activation. *Infect Immun*. **65**, 4005-4010

32. Forbes, S. J., Bumpus, T., McCarthy, E. A., Corthésy, B., and Mantis, N. J. (2011) Transient suppression of Shigella flexneri type 3 secretion by a protective O-antigen-specific monoclonal IgA. *MBio*. **2**, e00042-00011

33. Mathias, A., Longet, S., and Corthésy, B. (2013) Agglutinating secretory IgA preserves intestinal epithelial cell integrity during apical infection by Shigella flexneri. *Infect Immun*. **81**, 3027-3034

34. Renegar, K. B., Floyd, R. A., and Krueger, J. M. (1998) Effects of short-term sleep deprivation on murine immunity to influenza virus in young adult and senescent mice. *Sleep*. **21**, 241-248

35. Stubbe, H., Berdoz, J., Kraehenbuhl, J. P., and Corthésy, B. (2000) Polymeric IgA is superior to monomeric IgA and IgG carrying the same variable domain in preventing Clostridium difficile toxin A damaging of T84 monolayers. *J Immunol*. **164**, 1952-1960

36. Blander, J. M., and Sander, L. E. (2012) Beyond pattern recognition: five immune checkpoints for scaling the microbial threat. *Nat Rev Immunol*. **12**, 215-225

37. Corthésy, B. (2013) Multi-faceted functions of secretory IgA at mucosal surfaces. *Front Immunol*. **4**, 185

38. Perrier, C., Sprenger, N., and Corthésy, B. (2006) Glycans on secretory component participate in innate protection against mucosal pathogens. *J Biol Chem*. **281**, 14280-14287

39. Clerc, P., and Sansonetti, P. J. (1987) Entry of Shigella flexneri into HeLa cells: evidence for directed phagocytosis involving actin polymerization and myosin accumulation. *Infect Immun*. **55**, 2681-2688

40. Watarai, M., Funato, S., and Sasakawa, C. (1996) Interaction of Ipa proteins of Shigella flexneri with alpha5beta1 integrin promotes entry of the bacteria into mammalian cells. *J Exp Med*. **183**, 991-999

41. Norrby-Teglund, A., Ihendyane, N., Kansal, R., Basma, H., Kotb, M., Andersson, J., and Hammarstrom, L. (2000) Relative neutralizing activity in polyspecific IgM, IgA, and IgG preparations against group A streptococcal superantigens. *Clin Infect Dis*. **31**, 1175-1182

42. Singh, K., Chang, C., and Gershwin, M. E. (2013) IgA deficiency and autoimmunity. *Autoimmun Rev*.

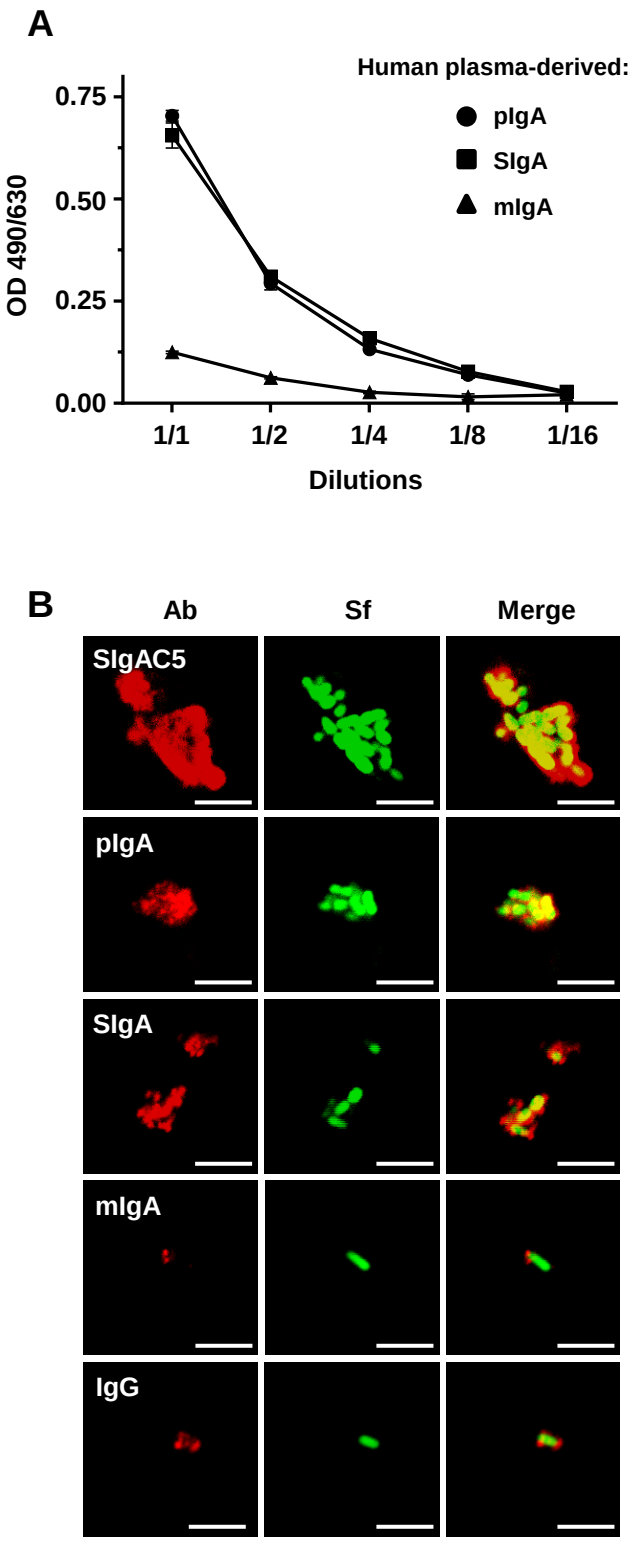
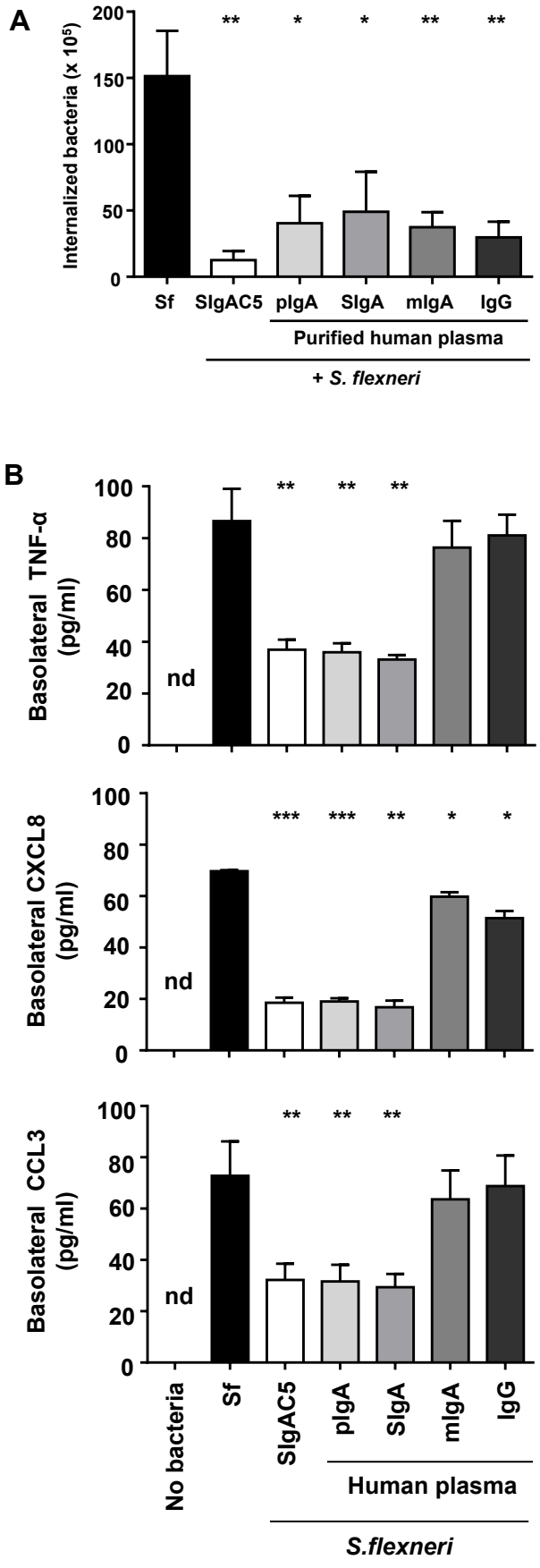

A
Human plasma-derived:
pIgA
SIgA
mIgA
OD 490/630
0.75
0.50
0.25
0.00
1/1
1/2
1/4
1/8
1/16
Dilutions
B
Ab
Sf
Merge
SIgAC5
pIgA
SIgA
mIgA
IgG

Figure 1


A
Internalized bacteria (x 10⁵)
200
150
100
50
0
**
*
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**
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Sf
SIgAC5
pIgA
SIgA
mIgA
IgG
Purified human plasma
+ S. flexneri
B
Basolateral TNF-α (pg/ml)
100
80
60
40
20
0
nd
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Basolateral CXCL8 (pg/ml)
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*
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Basolateral CCL3 (pg/ml)
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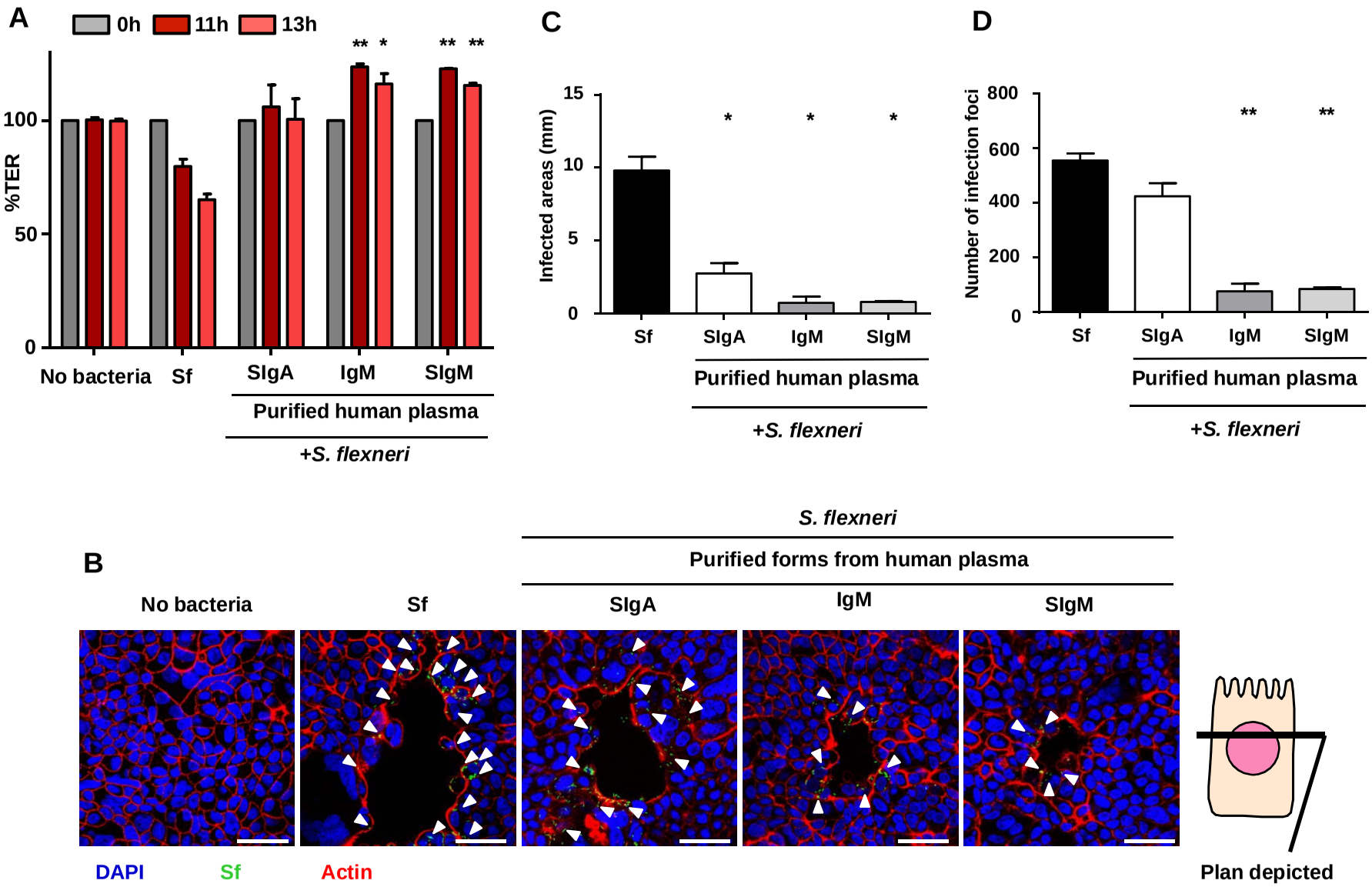
No bacteria
Sf
SIgAC5
pIgA
SIgA
mIgA
IgG
Human plasma
S.flexneri

Figure 2


A
0h
11h
13h
%TER
100
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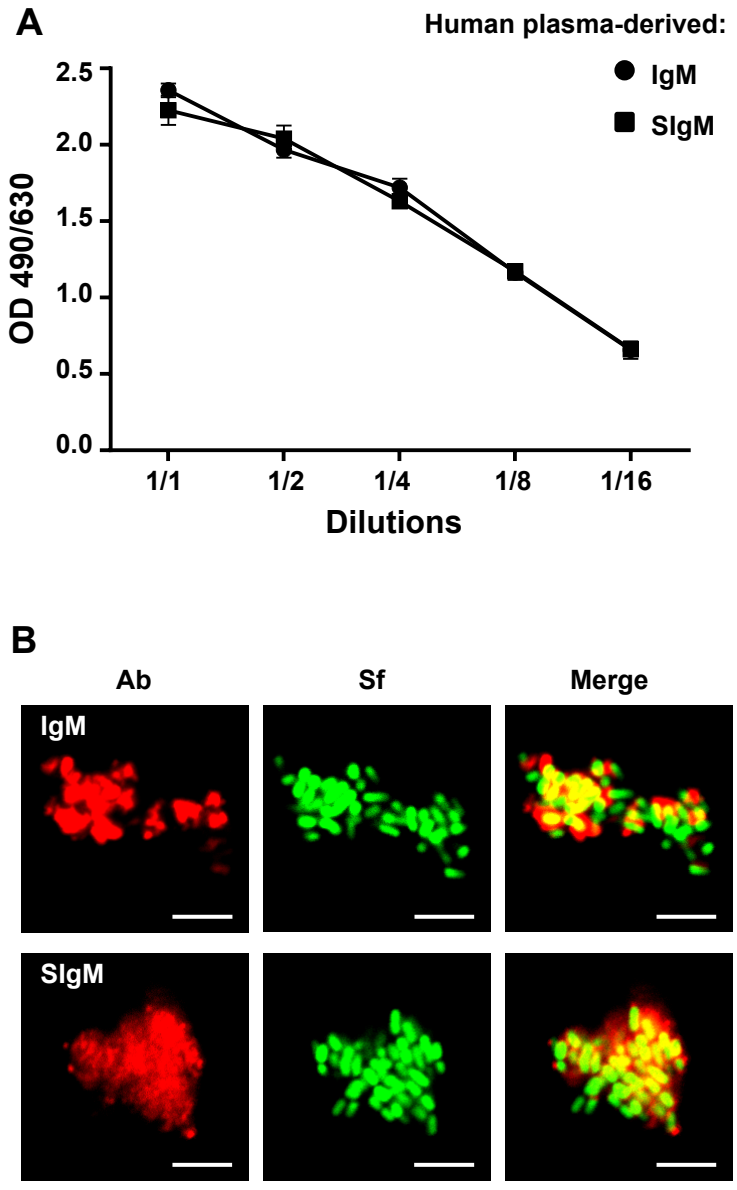
** * ** **
No bacteria
Sf
SIgA
IgM
SIgM
Purified human plasma
+S. flexneri
C
Infected areas (mm)
15
10
5
0
*
*
*
Sf
SIgA
IgM
SIgM
Purified human plasma
+S. flexneri
D
Number of infection foci
800
600
400
200
0
**
**
Sf
SIgA
IgM
SIgM
Purified human plasma
+S. flexneri
B
S. flexneri
Purified forms from human plasma
No bacteria
Sf
SIgA
IgM
SIgM
DAPI
Sf
Actin
Plan depicted

Figure 3


A
Human plasma-derived:
IgM
SIgM
OD 490/630
2.5
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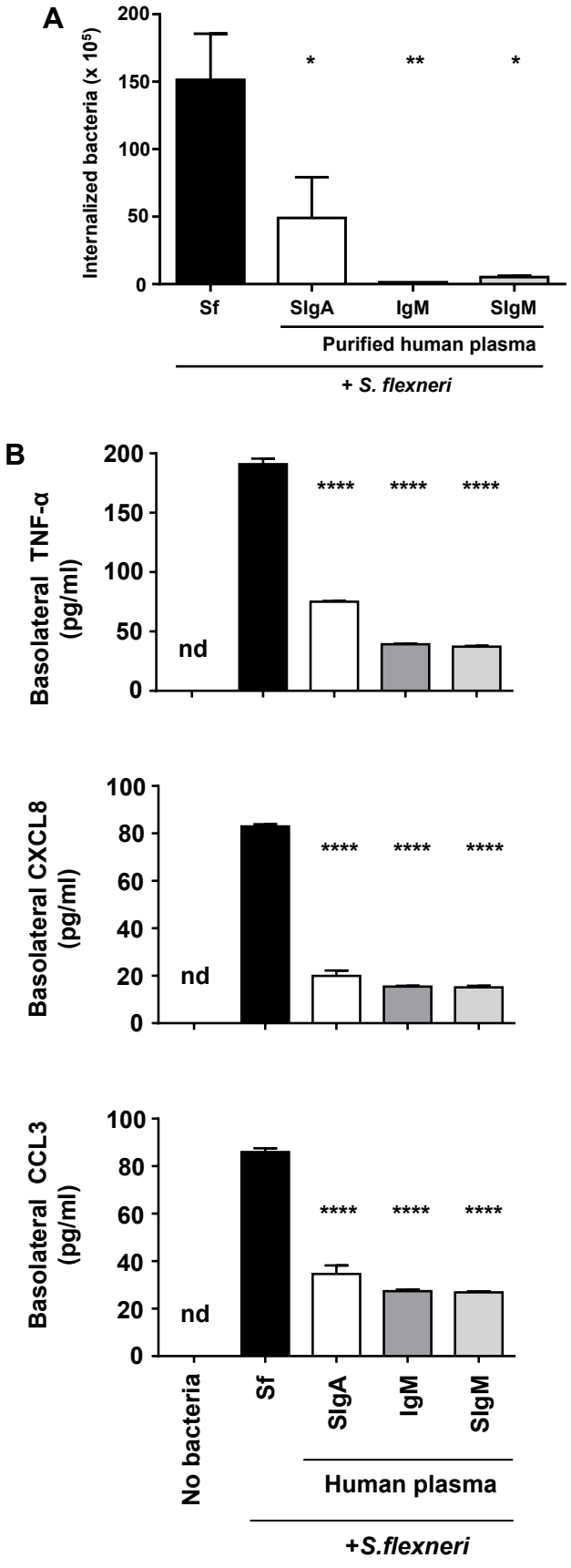
Dilutions
B
Ab
Sf
Merge
IgM
SIgM

Figure 4


A
Internalized bacteria (x 10⁵)
200
150
100
50
0
*
**
*
Sf
SIgA
IgM
SIgM
Purified human plasma
+ S. flexneri
B
Basolateral TNF-α (pg/ml)
200
150
100
50
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nd
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Basolateral CXCL8 (pg/ml)
100
80
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Basolateral CCL3 (pg/ml)
100
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No bacteria
Sf
SIgA
IgM
SIgM
Human plasma
+S.flexneri

Figure 5

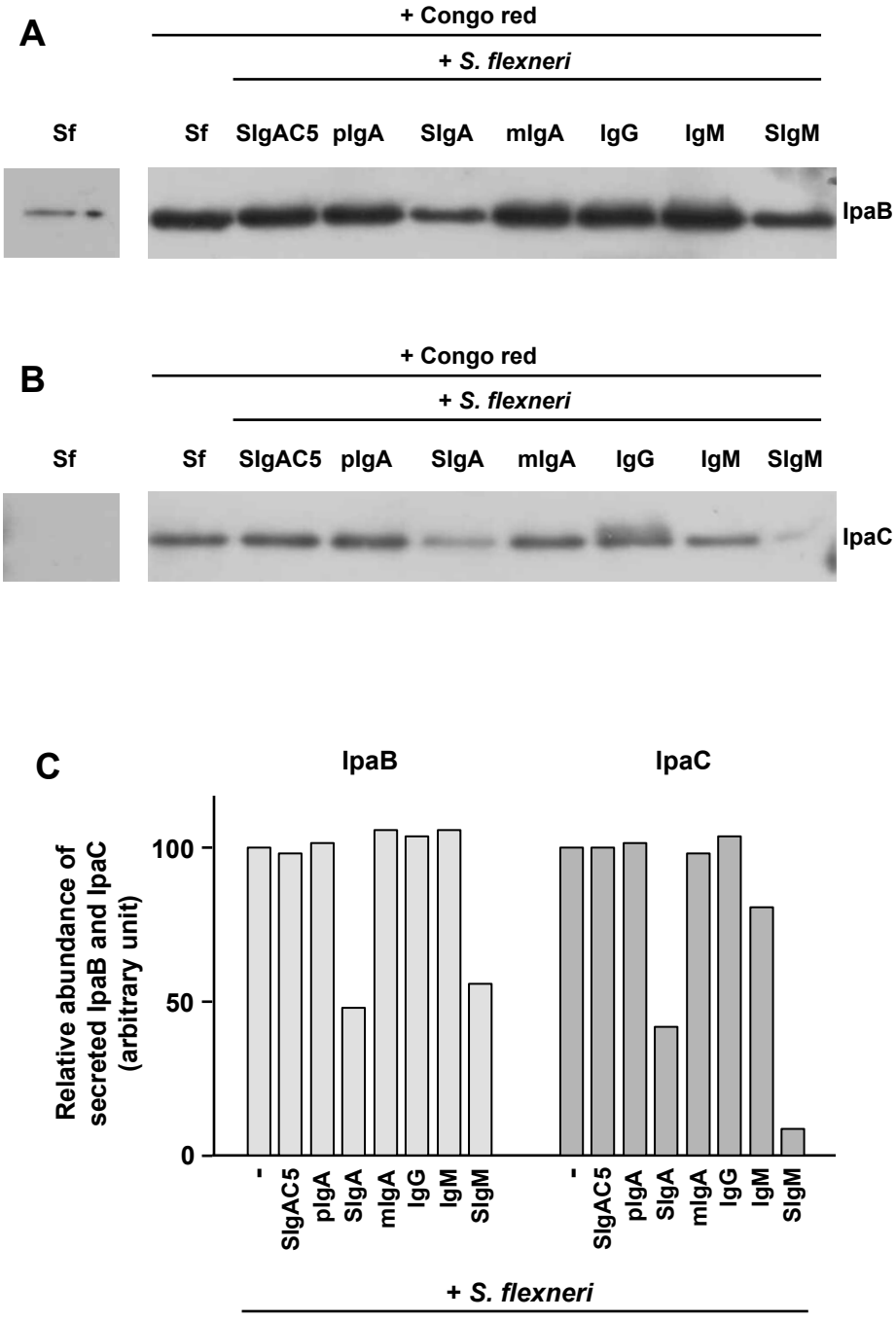
A
+ Congo red
+ S. flexneri
Sf
Sf
SIgAC5
pIgA
SIgA
mIgA
IgG
IgM
SIgM
IpaB
B
+ Congo red
+ S. flexneri
Sf
Sf
SIgAC5
pIgA
SIgA
mIgA
IgG
IgM
SIgM
IpaC
C
IpaB
IpaC
Relative abundance of secreted IpaB and IpaC (arbitrary unit)
100
50
0
-
SIgAC5
pIgA
SIgA
mIgA
IgG
IgM
SIgM
+ S. flexneri

Figure 6

Part III: Human plasma IgA-enriched preparations are functional against *Clostridium difficile* toxin A

Introduction

C. difficile is a gram-positive bacterium that is the primary cause of nosocomial antibiotic-associated diarrhea and colitis. Indeed, *C. difficile* proliferates in the intestine of patients with a disrupted microbiota[86]. The current treatment relies on antibiotics to eradicate *C. difficile* infection. However, relapse of disease is a continuing challenge[194]. Consequently, other strategies to fight these infections need to be developed. As pathogenicity is based on toxins, especially toxin A, secreted by this bacterium, and as human plasma polyreactive IgA showed remarkable protective effects against an enteropathogenic bacterium[141], the functionality of human plasma IgA was then explored against *C. difficile* toxin A using intestinal epithelial Caco-2 cell monolayers. Protection of cell monolayers was assessed by monitoring of TER, confocal microscopy analysis and measurement of cell monolayer inflammation. We found that human plasma IgA-enriched preparations containing pIgA or reconstituted SIgA led to a remarkable maintenance of TER and a diminution of cell monolayer damage for up to 36 h post-exposure to the toxin. Finally, we demonstrated that these IgA-enriched preparations allowed a reduction of pro-inflammatory mediator secretion by cell monolayers. The sum of these results shows that these molecules are functional against toxin A. Further studies should be performed to dissect the mode of action of these molecules against toxin A.

Materials and methods

Preparation of human plasma IgA-enriched fractions - IgA molecules were purified from process intermediates of immunoglobulins manufactured from human plasma by affinity chromatography using CaptureSelect Human IgA (Bioaffinity Company BAC)[184]. The starting material used was a chromatography side fraction consisting of the strip fraction from an ion-exchange chromatography column used in the large scale manufacture of IgG from human plasma. The different starting materials were diluted in phosphate buffered saline (PBS) to IgA concentration of approximately 1 mg/ml and then loaded onto a CaptureSelect Human IgA column pre-equilibrated with PBS, without exceeding the IgA-binding capacity of the column. After loading, the column was washed with PBS and IgA was eluted with glycine buffer at pH 3.0. The eluate was adjusted with 0.5 M Tris-base (pH 8.0) to pH 4.5 and concentrated up to 16 mg/ml protein.

Separation of plasma-derived pIgA and mIgA - IgA-enriched preparations containing a mixture of mIgA and pIgA were diluted in PBS to a final volume of 10 ml suitable for injection onto the ÄKTA*prime* chromatography system (GE Healthcare). The flow rate was set at 1 ml/min with PBS as mobile phase for all runs. Separation of the two molecular forms of IgA was performed on two serially coupled 1-meter long columns filled with Sephacryl S-300 HR beads[195]. The IgA content of 3.5-ml fractions was verified by immunodetection using polyclonal rabbit anti-human IgA/HRP (1/3000, Dako) and pools of mIgA and pIgA were obtained. Concentration was performed using the Labscale system (Millipore) connected to a 100-kDa cut-off cartridge, and stored at 4 °C until further use.

Proteins - Chimeric mouse-human pIgA PCG-4 specific for *C. difficile* toxin A were produced and purified as described[49]. Recombinant hSC was produced from a Chinese hamster ovary clone stably transfected with an expression cassette coding for the protein[61]. *C. difficile* toxin A was purchased from Calbiochem. Lyophilised toxin A was gently resuspended in H_2O just before using it. Note: a new provider of toxin A should be found because problems related to toxin activity were encountered during this thesis work.

In vitro reassociation of polymeric IgA and hSC - SIgA molecules were obtained by combining *in vitro* human plasma-derived pIgA molecules purified or contained in IgA-enriched preparations with hSC (referred as SIgA or SIgA in IgA-enriched preparations, respectively all along the text) with a stoichiometry 1:1 in PBS for 30 min at room temperature (RT) as previously described[59]. The reassociation was verified by immunodetection[141].

Caco-2 cell culture and growth as polarized monolayer - The human colonic adenocarcinoma epithelial Caco-2 cells (American Type Tissue Collection) were grown in complete Dulbecco's modified eagle medium (C-DMEM) consisting of DMEM-Glutamax (Invitrogen) supplemented with 10% fetal calf serum (FCS, Sigma), 10 mM HEPES (Invitrogen), 1% non essential amino acids (Gibco), 1% Sodium Pyruvate (Gibco), 1% L-glutamine (Sigma), 1% penicillin/streptomycin (Sigma) and 0.1% transferrin (Invitrogen). The cells were seeded on polyester Snapwell filters (diameter, 12 mm; pore size, 0.4 µm; Corning Costar) as previously described[196]. The Caco-2 cell monolayer integrity was checked by measuring the TER using Millicell-ERS device (Millipore)[197].

Formation of immune complexes - 0.3 nM of toxin A was mixed with pIgA PCG-4 (7.5 nM) or with human plasma pIgA, SIgA or mIgA (1.82 µM). Toxin A was also mixed with pIgA or SIgA (1.82 µM) in IgA-enriched preparations (also containing 11.27 µM of mIgA). All mixtures were prepared in a final volume of 500 µl of plain DMEM (p-DMEM: DMEM complemented with 10 mM HEPES (Invitrogen), 1% non essential amino acids (Gibco), 1% Sodium Pyruvate (Gibco), 1% L-glutamine (Sigma) and 0.1% transferrin (Invitrogen)). The mixtures were incubated for 30 min at RT under gentle agitation.

Protection assay - 1 h before the use of polarized Caco-2 cell monolayers, C-DMEM was replaced by P-DMEM in both the apical and basolateral compartments. Polarized Caco-2 cell monolayers were infected apically with *C. difficile* toxin A alone or in combination with the Ab preparations. Exposure of Caco-2 cells to antigens or the various immune complexes was performed for up to 36 h and toxin A-induced damages were tracked by measuring TER decrease timewise.

Laser-scanning confocal microscopy (LSCM) observation of Caco-2 cell monolayers - To examine the integrity of Caco-2 cell monolayers, Snapwells were washed twice with PBS, prior to fixation overnight with 5 ml of 4% paraformaldehyde at 4 °C. After washing, filters were permeabilized and non-specific binding sites were blocked using PBS containing 5% FCS and 0.2% Triton X-100 for 30 min at RT. All Abs were diluted in this solution. Filters were incubated with rabbit anti-human ZO-1 (1/200, Invitrogen) for 2 h at RT, washed in PBS, followed by goat anti-rabbit IgG conjugated with Alex Fluor® 647 (1/100, Invitrogen) for 90 min at RT. To visualize the actin network, Phalloidin associated with Fluoroprobes 547H (1/200, Interchim) was incubated concomitantly with the secondary Ab. To visualize cells, filters were finally incubated with 200 ng/ml of 4',6-diamidino-2-phenylindole (DAPI, Invitrogen) in PBS for 30 min. Filters were cut out of their holders, and mounted in Vectashield solution for observation using Zeiss LSM 710 Meta confocal microscope (Carl Zeiss, Germany) equipped with 40x objective. 3D reconstructions along the xy plans, as well as orthogonal projections along the z plan were performed using the Zeiss ZEN 2009 light software.

Enzyme-linked immunosorbent assay (ELISA) - Human CXCL8, GRO-α and MCP-1 concentrations in the basolateral compartment of polarized Caco-2 cell monolayers infected by *C. difficile* toxin A alone or combined to Abs were measured by ELISA using commercial kits (BD Biosciences and R&D Systems, respectively).

Results

Human plasma IgA-enriched preparations reduce damage of Caco-2 cell monolayers exposed to C. difficile toxin A

In order to examine if polyspecific human plasma-derived IgA could be functional against *C. difficile* toxin A, Caco-2 cell monolayers were exposed to *C. difficile* toxin A alone or complexed to human plasma IgA. The range of concentrations of purified pIgA and SIgA protecting Caco-2 cell monolayers against *S. flexneri* failed to protect monolayers from toxin A exposure (data not shown). Consequently, we decided to consider the protective effect of IgA-enriched preparations containing higher concentrations of IgA in order to demonstrate toxin A neutralization. Several concentrations of IgA-enriched preparations used as such or complemented with hSC were evaluated in comparison with anti-toxin A protective pIgA PCG-4 by monitoring TER changes. Similar maintenance of TER was obtained with 7.5 nM of pIgA PCG-4 and with 18, 6.07, 3.64 and 1.82 µM of human plasma pIgA or SIgA in IgA-enriched preparations up to 28 h post-exposure to the toxin (data not shown). In order to further evaluate the role of the various molecular forms in this protective effect, 1.82 µM of purified human plasma pIgA, SIgA and mIgA were evaluated in comparison with the same concentrations of pIgA and SIgA included in IgA-enriched preparations. A significant and long-term maintenance of TER was observed with pIgA and SIgA in IgA-enriched preparations, whereas toxin A alone or complexed with purified mIgA led to a drop of TER within 36 h post-exposure. Even though a drop of TER was observed with purified pIgA and SIgA, the decrease of TER was delayed as compared to the one observed with toxin A alone (**Figure 1**). This difference was close to be significant at numerous time-points.

In order to examine the integrity of Caco-2 cell monolayers, LSCM observations (snapshots) of cell monolayers were performed 36 h after exposure to toxin A alone or associated with 1.82 µM of purified pIgA, SIgA or mIgA, as well as the same concentration of pIgA or SIgA in enriched-preparations. Purified pIgA and SIgA, as well as pIgA and SIgA in enriched-preparations similarly reduced the areas devoid of nuclei compared to the condition with toxin A alone. While the actin network shown via phalloidin

labeling was preserved under all conditions, tight junction network visualized via ZO-1 labeling was similarly maintained with purified pIgA and SIgA, as well as IgA-enriched preparations. Consistent with data of TER measurement, purified mIgA did not preserve the integrity of the monolayer. Even though the areas devoid of nuclei are reduced, distortions of the tight junction network suggest significant damages of cell monolayers (**Figure 2**). The sum of this data shows that human plasma IgA-enriched preparations, as well as purified pIgA and SIgA to a lesser extent, are able to reduce damage of cell monolayers induced by toxin A incubation.

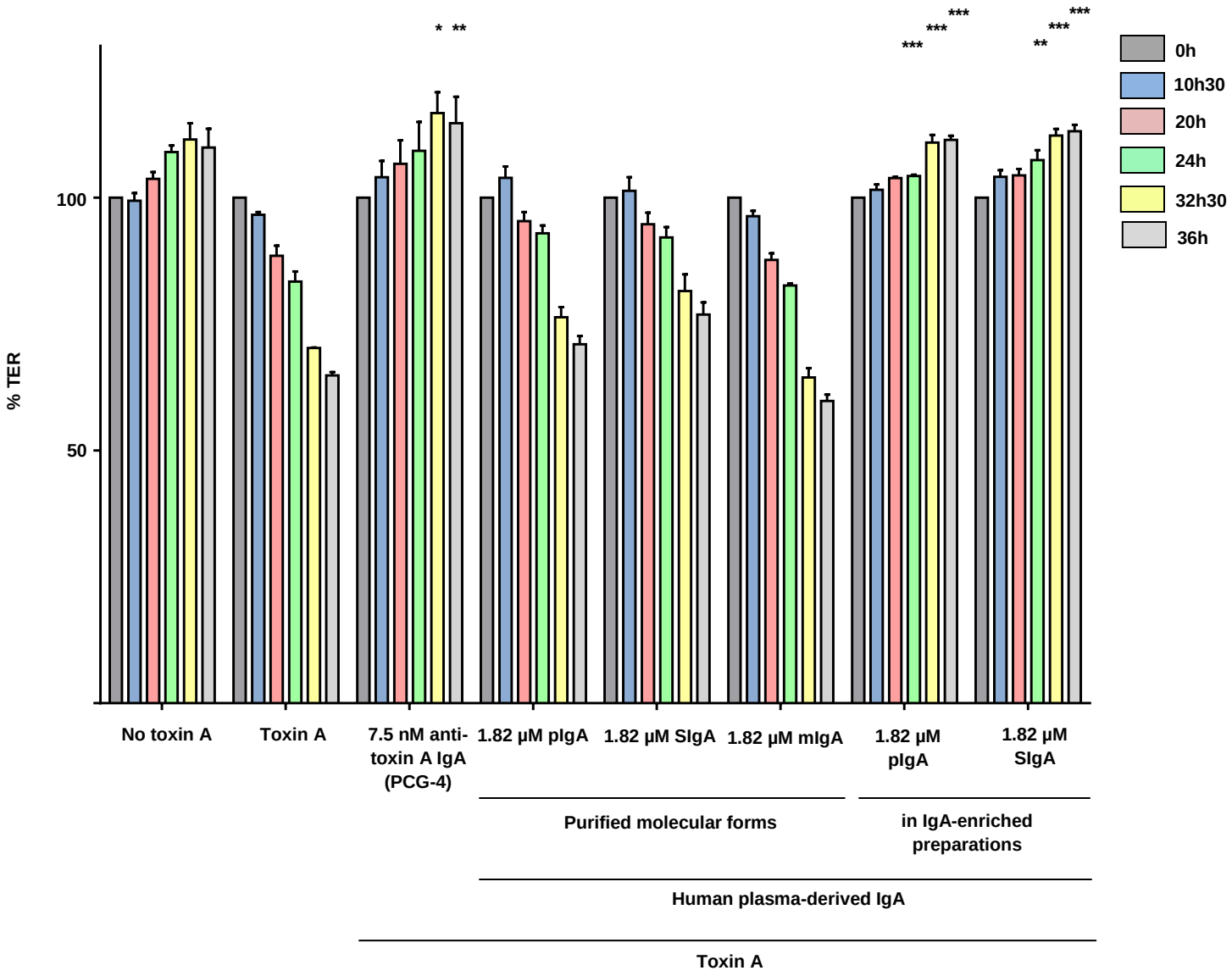

Figure 1: Monitoring of TER changes of Caco-2 cell monolayers exposed to *C. difficile* toxin A alone or complexed with human plasma IgA. TER of intestinal Caco-2 cell monolayers exposed to 0.3 nM of toxin A alone or in combination with purified human plasma pIgA, SIgA, mIgA, or pIgA, SIgA in IgA-enriched preparations determined at six time-points. The TER values for each condition and each time-point were normalized to the TER values at the beginning of the experiment and are expressed in percentage. Protection offered by chimeric anti-toxin A pIgA PCG-4 and non-infected Caco-2 cell monolayers (No toxin A) serve as controls. The panel is representative of one individual experiment performed in triplicates. Mean values + SEM are shown. Significant statistical differences calculated by comparison with the condition toxin A alone (Toxin A) are indicated above the columns: *p<0.05; **p<0.01; ***p<0.001.

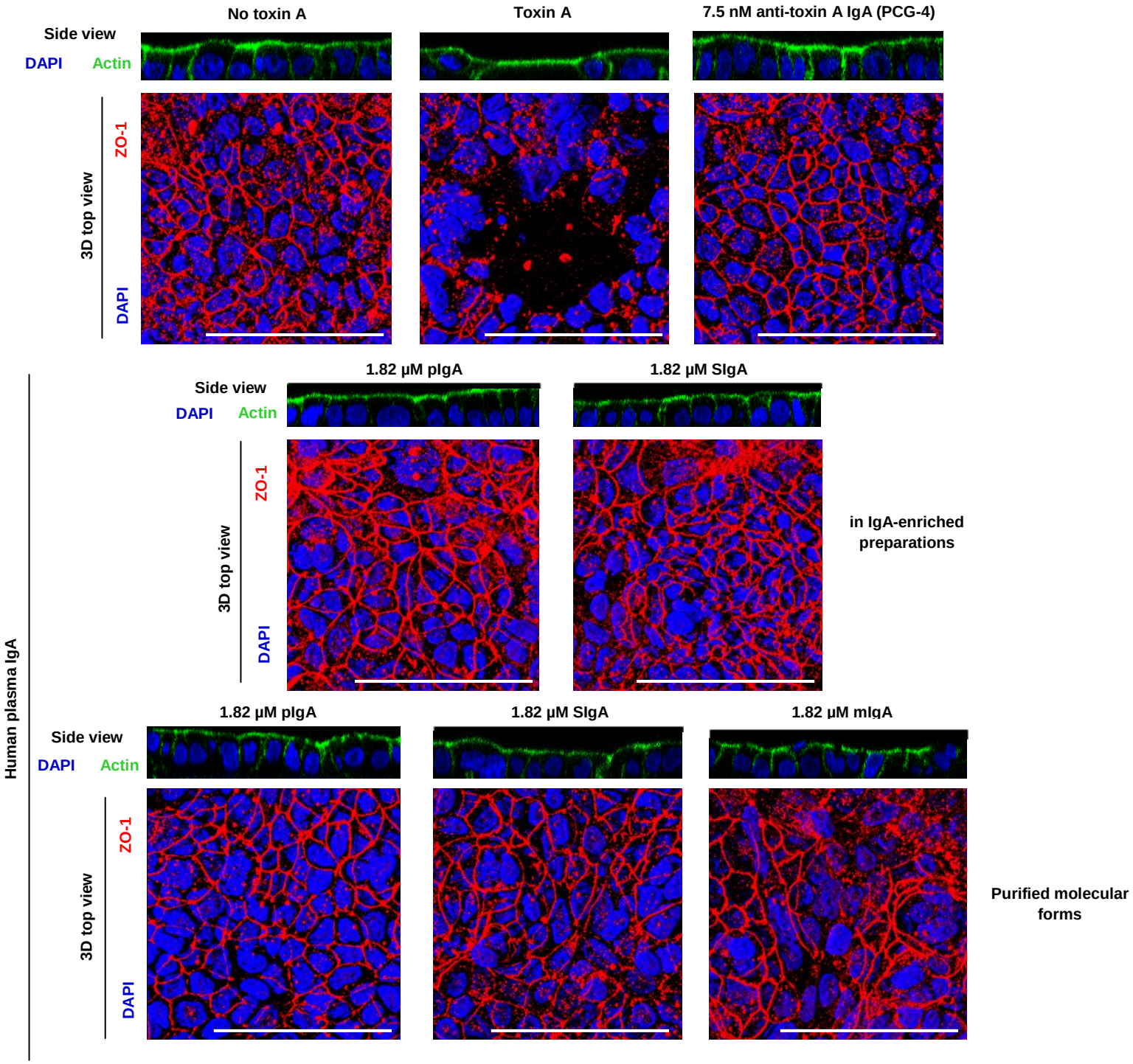

Figure 2: LSCM observations of Caco-2 cell monolayers exposed to *C. difficile* toxin A alone or in combination to human plasma IgA. LSCM 3D reconstructed images (snapshots) of Caco-2 cell monolayers exposed to 0.3 nM of toxin A alone or in combination with human plasma-derived pIgA, SIgA or mIgA, as well as pIgA or SIgA in IgA-enriched preparations for 36 h. Tight junctions stabilizing the monolayer are visualized by ZO-1 labeling (3D top view, red) and actin network is visualized by phalloidin labeling (side views, green). Caco-2 cells are visualized via nuclear staining with DAPI (blue). Protection offered by chimeric anti-toxin A pIgA PCG-4 and non-infected Caco-2 cell monolayers (No toxin A) serve as controls. The panel is representative of one individual experiment. Scale bars: 50 µm.

Human plasma IgA-enriched preparations diminish the secretion of pro-inflammatory mediators by cell monolayers

Damages of cell monolayers in presence of toxin A were especially reduced with 1.82 µM of pIgA or SIgA in enriched-preparations. In order to investigate if these IgA-enriched preparations impact on the sensing of Caco-2 cell monolayers to toxin A, cell monolayers were exposed to toxin A alone or associated to 1.82 µM of pIgA or SIgA in enriched preparations for 36 h. Specific pIgA PCG-4 was used as positive control. The basolateral secretion of MCP-1[92], GRO-α[92] and CXCL8[91] was measured by ELISA. Similarly to specific IgA PCG-4, pIgA and SIgA in IgA-enriched preparations led to a significant decrease of MCP-1, GRO-α and CXCL8 secretion by cell monolayers compared to the condition with toxin A alone (**Figure 3**). Therefore, human plasma IgA-enriched preparations are able to reduce inflammation of cell monolayers caused by toxin A exposure. These results suggest a neutralization of toxin A and a subsequent diminution of contacts between toxin A and Caco-2 cell monolayers.

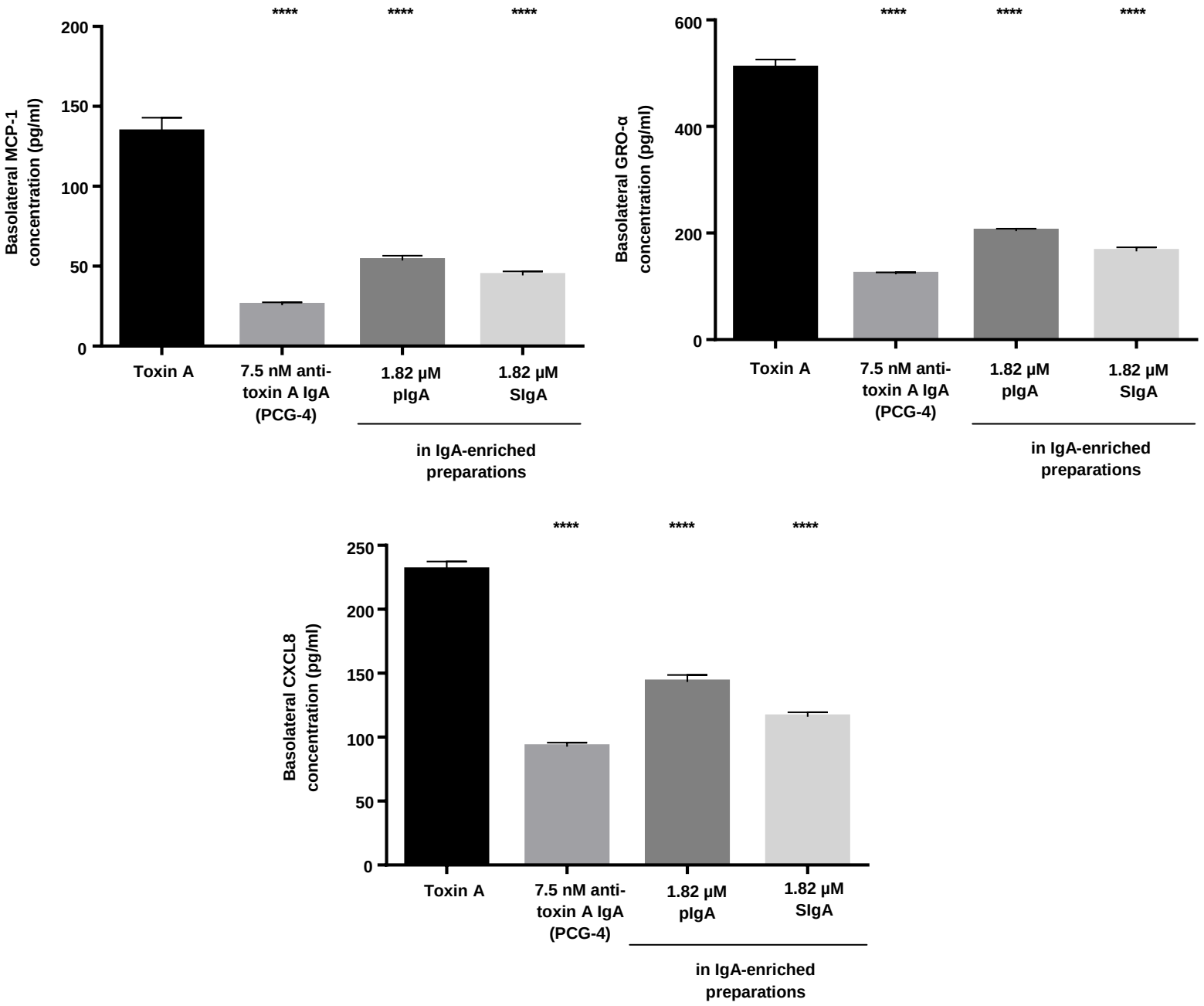

Figure 3: Secretion of MCP-1, GRO-α and CXCL8 by Caco-2 cell monolayers exposed either to *C. difficile* toxin A alone or complexed with human plasma IgA. Caco-2 cell monolayers exposed to 0.3 nM of *C. difficile* toxin A alone or in combination with human plasma-derived pIgA or SIgA in IgA-enriched preparations for 36 h. At this time-point, secretion of MCP-1, GRO-α and CXCL8 was measured in the medium of the basolateral compartment of Caco-2 cell monolayers. Data of two experiments performed in triplicates (n=6). Data obtained with chimeric anti-toxin A pIgA PCG-4 serve as control. Mean values + SEM are shown. Significant statistical differences calculated by comparison with the condition *C. difficile* toxin A alone (Toxin A) are indicated above the columns: ****p<0.0001.

Discussion

In this study, we have demonstrated that IgA-enriched preparations efficiently maintained the integrity of Caco-2 cell monolayers and reduced their inflammatory responses in presence of *C. difficile* toxin A for up to 36 h post-exposure. These data underscore the protective effect of human plasma polyclonal IgA against an enteropathogenic toxin and are consistent with data described by Johnson et al., (1995)[99]. Indeed, this study demonstrated that serum IgA neutralized the cytotoxicity of toxin A on cells *in vitro* but also in a rabbit ileal loop model. Strikingly, higher concentrations of human plasma IgA have to be used compared to the ones needed to protect cell monolayers from infection by *S. flexneri*. This difference of IgA concentration required for protection may be due to the fact that more epitopes can be targeted by polyreactive IgA in the case of a whole bacterium than for a toxin alone.

Stubbe and colleagues (2000)[49] showed that specific anti-toxin A pIgA was superior to mIgA carrying the same variable domain to protect polarized intestinal cell monolayers from *C. difficile* toxin A. In this present study using polyreactive Abs, purified pIgA and SIgA led to a better maintenance of TER than purified mIgA but this difference was not significant. Tight junction network of cell monolayers was also slightly better maintained with purified pIgA and SIgA than with mIgA. However, the best protection of cell monolayers was obtained with pIgA and SIgA in enriched-preparations that contain also numerous mIgA molecules. The protective effect may be due to the additive effects of mIgA and pIgA or SIgA molecules. But it is also crucial to keep in mind that in these IgA-enriched preparations small amounts of IgG and IgM still remain. We cannot exclude that these molecules, especially IgM, do not contribute to the protective functions observed with these preparations.

Consistent with the data obtained in experiments dealing with *S. flexneri*, the presence of hSC did not modify the protective effect conferred by purified pIgA or pIgA in enriched-preparations. However, it is known that in the *in vivo* context, the presence of bound SC is crucial to the stability and anchoring of the molecule at mucosal surfaces[46]. In addition, Perrier et al. (2006)[63] showed that SC alone could protect Caco-2 cell monolayers from cytopathic effects of toxin A thanks to glycans present on it.

Further studies should be performed to unravel the modes of action of IgA-enriched preparations involved in their protective functions. Particularly, interaction between toxin A and human plasma IgA should be studied. Finally, the functionality of human plasma IgM against toxin A could be also evaluated using Caco-2 cell monolayers.

Patent: Compositions comprising secretory-like immunoglobulins

Parts I-III of this thesis manuscript are compiled in the published patent entitled:

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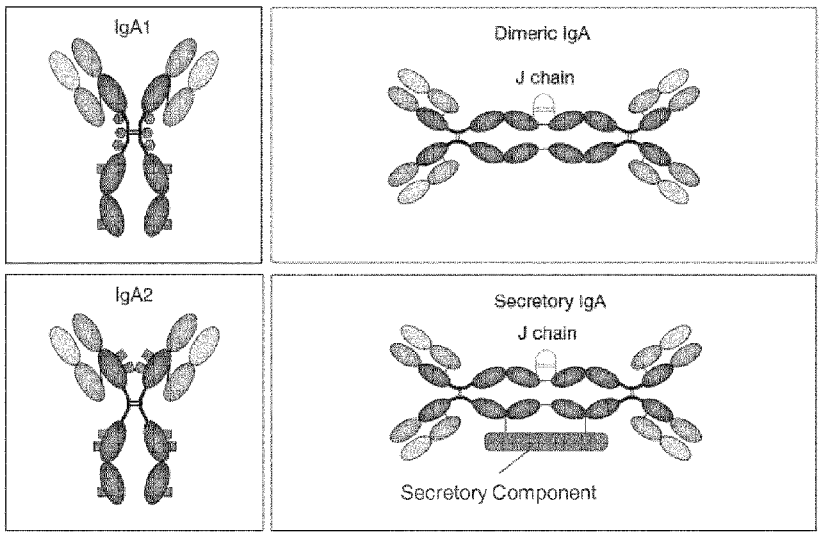
(54) Title: COMPOSITIONS COMPRISING SECRETORY - LIKE IMMUNOGLOBULINS

Figure 1



(57) Abstract: The invention relates to methods for preparing compositions comprising secretory- like immunoglobulin, in particular secretory-like IgA and/or secretory-like IgM, and compositions obtainable by the methods.

WO 2013/132052 A1

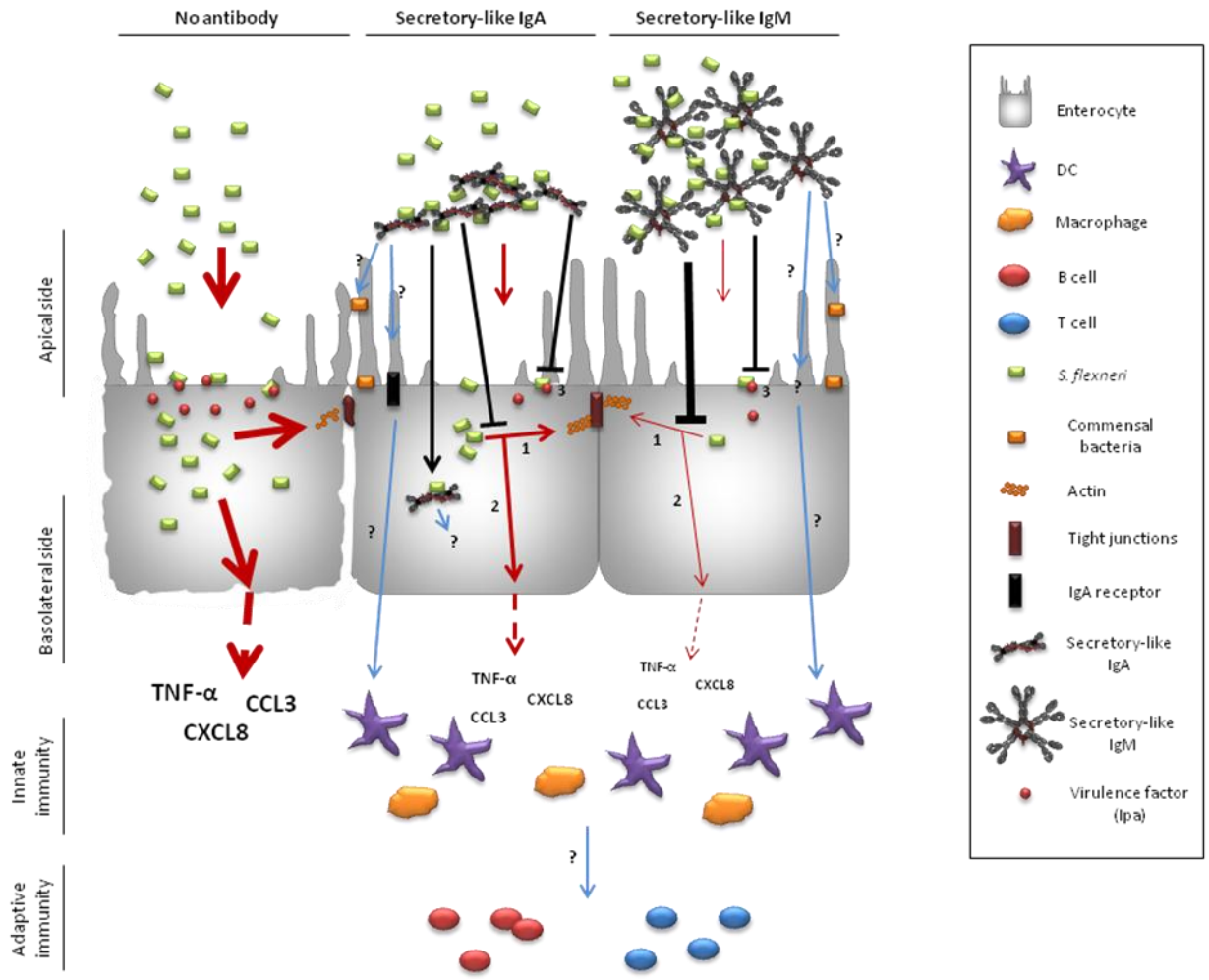


Figure 1: Demonstrated protective effects of secretory-like IgA and IgM and unknown aspects to be explored. At mucosal surfaces, secretory-like IgA and IgM prevent invasion of intestinal epithelium by *S. flexneri* via the formation of aggregated immune complexes. This leads to maintenance of epithelial cell integrity by preserving tight junctions and actin network (1), and a decrease of pro-inflammatory mediator secretion by epithelial cells (2). Both secretory-like Abs also act on bacterial virulence by diminishing virulence factor secretion by bacteria (3). The intracellular mechanisms involved in cellular sensing of bacteria-SIgA complexes have to be explored. In addition, the potential transport of immune complexes into the lamina propria and the subsequent impact on innate and adaptive immune cells remain in need of investigation. Finally, the influence of secretory-like Abs on commensal flora also remains to be explored. Red arrows: impacts of bacteria on cells. Red dashed arrows: reactions of cells to infection. Black lines: impact of antibodies. Blue arrows: questions to be addressed. Nuclei of cells are deliberately not depicted. Not drawn to scale.

Outlooks and concluding remarks

In this work, we have demonstrated that secretory-like IgA and IgM could be generated by associating polyreactive human plasma-derived pIgA and IgM with recombinant human SC. In addition, we established that pIgA and secretory-like IgA, as well as IgM and secretory-like IgM were functional to protect *in vitro* Caco-2 cell monolayers from *S. flexneri* infection. Indeed, via formation of aggregated immune complexes, these molecules led to maintenance of the epithelial cell integrity (**Figure 1: 1**), decrease of pro-inflammatory mediator secretion by epithelial cells (**Figure 1: 2**) and reduction of bacterial infectivity (**Figure 1: 3**). Finally, we reported that human plasma IgA molecules were also functional against *C. difficile* toxin A.

The next step will be to focus on the generation of an animal model to evaluate the functionality of human plasma IgA and IgM, as well as secretory-like IgA and IgM in the *in vivo* context. As the protective effects of the molecules were particularly highlighted in our *in vitro* model using *S. flexneri*, the use of a bacterium as model of pathogen will be favored in *in vivo* models. 1) An ileal loop mouse model to test the protective functions of secretory-like Abs would represent a first approach. Infection of ileal loops with *Salmonella* alone or *Salmonella* complexed to secretory-like Abs will be performed. The bacterial load of PPs will be numerated by plating to test whether the presence of Abs can reduce bacteria invasion of PPs. Additional read-outs to analyze infection such as confocal microscopy or analysis of cytokine expression by qPCR could also be considered. 2) An alternative mouse protection assay based on intranasal infection by *S. flexneri*[61] could also be considered. Intranasal infection by *S. flexneri* alone or in combination with secretory-like Abs will be performed and a few hours later the bacterial load of lungs will be assessed by plating, confocal microscopy and histological studies. Inflammatory response will be examined by measuring pro-inflammatory mediator secretion in bronchoalveolar lavages. This latter model could also be used to test the role of secretory-like Abs in prophylactic administration. 3) As alternative to mucosal application, i.v. administration of secretory-like Abs could also be considered. In

order to investigate if i.v. administration of human plasma IgA or IgM can protect from *Salmonella* infection, human plasma IgA or IgM will be injected i.v. into mice, the transport of human Abs into mucosal secretions will be analyzed and mice will be infected with *Salmonella* by oral gavage. *Salmonella* translocated into the SED in PPs will be quantified by confocal microscopy. In addition, plating of the PPs, MLNs and spleen will be also performed in order to numerate bacteria in these tissues and assess a potential modulation of bacterial load in mice administered with Abs. However, using these protection models, it is essential to keep in mind that murine endogenous secretory Abs may mask the effect of the exogenously delivered secretory-like Abs. Several of these analyses have been initiated during the last months of the thesis work.

It will be also crucial to study the modes of action of secretory-like Abs using *in vivo* models. On the one hand, the results obtained by *in vitro* experiments performed in this thesis work suggest that immune exclusion should be the main mechanism involved in protection against microbial infections at mucosal surfaces (**Figure 1**). This mode of action could even be increased by the presence of additional partners found in an *in vivo* context such as mucus[46]. In this context, the type of interactions between secretory-like Abs and antigens ($F(ab')_2$ or Fc-dependent) and the involvement of carbohydrates in interactions remain to be explored. On the other hand, our *in vitro* data also suggest additional cellular mechanisms involved in protection, especially with IgA. Whether these polyreactive secretory-like Abs form immune complexes that can be internalized *in vivo* by PP DCs and whether once entered, these immune complexes are able to down-regulate inflammatory circuits induced by an infection, as demonstrated with specific SIgA[67,198], has to be determined. This process can possibly take place with secretory-like IgA as a selective IgA receptor described on M cells was shown to interact with plasma IgA[121,122]. Similar mechanisms for IgM have not been described. Whether polyreactive secretory-like Abs are able to lead changes in endogenous microflora, e.g. improve adherence of some commensal bacterial strains, has to be explored. Polyreactive human milk-derived SIgA was shown to increase adherence of some commensal bacteria and formation of biofilm in the gut, we may hypothesize that such changes could be observed with secretory-like Abs[199]. In

a potential context of i.v. administration, additional mechanisms involving the binding of the molecules to IgA and IgM receptors found on various immune cells could take place. Like endogenous Abs, human plasma IgA and/or IgM could bind Fc receptors such as FcαRI[36], Fcα/μR[111], FcμR[178] or other Fc receptors and potentially could modulate cellular and inflammatory responses.

In addition to intrinsic functions of IgA and IgM, the great advantage of human plasma-derived Abs is their polyspecificity. Contrary to monoclonal Abs (e.g. IgAC5 directed against *S. flexneri* 5a LPS), these molecules target numerous epitopes of many pathogens. Therefore, numerous types of infection could be fought using these molecules. The results obtained in this work suggest that the amount of polyreactive Abs required to protect from infections varies according to the type of antigens/microorganisms involved in infection. As compared to IVIg administration (ca. 0.4g/kg), it may be hypothesized that mucosal application of secretory-like IgA/IgM could lead to reduce the dose of administered molecules because these ones would be directly delivered at the effector site of infection. Moreover, the type of infections will likely also impact on the administration route, e.g. to prevent gastrointestinal infections, oral route could be favored, whereas in case of respiratory tract infection, intranasal administration could be chosen. However, in case of sepsis, i.v administration could be preferred in order to act at mucosal and systemic levels. Finally, another important aspect to consider is how long the molecules will be detected at mucosal and/or systemic levels after administration. The obtained results will probably also impact on the administered dose and the administration intervals in case of a potent future clinical application.

Related to the potential administered doses in clinical applications, the amount of generated secretory-like Abs will not be a limiting factor. Indeed, pIgA purified from 10,000 liters of human plasma will provide enough Abs for clinical application[141]. In addition, recombinant human SC is produced by Chinese hamster ovary clones generated under good manufacturing practice conditions and resulting in sufficient amounts of SC for the association with purified pIgA.

Another important point towards clinical applications is the potential adverse effects of Ig preparations. Probably similar adverse effects observed with plasma IgG preparations could be expected with plasma

IgA and IgM[190] even though they could depend on the administration route. In addition, the structure of polymeric IgA and IgM may impact on the formation of aggregates in the preparations compared to IgG preparations, thus increasing the risk of innate immune cell activation and subsequent cytokine storm induction. This risk could be favored by a systemic administration of the molecules therefore, it is an additional reason why mucosal application is preferred.

In this PhD thesis, the use of polyreactive human plasma secretory-like Abs were successfully tested against mucosal infections and the decrease of pro-inflammatory mediator secretion observed in several experiments favors the evaluation of these molecules using *in vitro* and *in vivo* models of mucosal inflammations.

Knowing the various functions of IgA and IgM and the results presented here, secretory-like Abs obviously appear to be promising molecules to consider among therapeutic Abs. Their application in humans would certainly allow to target a very large panel of mucosal infections and inflammations.

Significant contribution to an additional research project

I contributed to a project aiming at dissecting the modes of action of SIgAC5 monoclonal Ab specific for *S. flexneri* LPS. The molecular mechanisms involved in the maintenance of epithelial integrity were investigated using polarized Caco-2 cell monolayers as model of intestinal epithelium. Caco-2 cell monolayers were infected by *S. flexneri* alone or complexed to SIgAC5. Damage and inflammatory responses of cell monolayers were tracked.

Experimental procedures, results, discussion

The results obtained in this part are compiled in the paper published in Infection and Immunity and entitled:

Agglutinating secretory IgA preserves intestinal epithelial cell integrity during apical infection by Shigella flexneri. Mathias A, Longet S, Corthésy B. Infect Immun. 2013 Aug;81(8):3027-34.

Take-home messages of the paper

- Interaction of virulent *S. flexneri* with the apical pole of polarized Caco-2 cell monolayers led to a disorganization of tight junctions and actin network, as well as cell death
- Agglutinating features of anti-LPS SIgAC5 Ab conducted to a delay in this disruption process
- Neutralization of *S. flexneri* by SIgAC5 interfered with cellular responsiveness: decrease in NF-κβ nuclear translocation and cytokine/chemokine release
- Similar results were not observed with monomeric IgAC5 and IgGC20 monoclonal Abs of the same specificity or SIgASal4 specific to *Salmonella typhimurium*

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Agglutinating Secretory IgA Preserves Intestinal Epithelial Cell Integrity during Apical Infection by *Shigella flexneri*

Amandine Mathias, Stéphanie Longet, Blaise Corthésy

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***Shigella flexneri*, by invading intestinal epithelial cells (IECs) and inducing inflammatory responses of the colonic mucosa, causes bacillary dysentery. Although M cells overlying Peyer's patches are commonly considered the primary site of entry of *S. flexneri*, indirect evidence suggests that bacteria can also use IECs as a portal of entry to the lamina propria. Passive delivery of secretory IgA (SIgA), the major immunoglobulin secreted at mucosal surfaces, has been shown to protect rabbits from experimental shigellosis, but no information exists as to its molecular role in maintaining luminal epithelial integrity. We have established that the interaction of virulent *S. flexneri* with the apical pole of a model intestinal epithelium consisting of polarized Caco-2 cell monolayers resulted in the progressive disruption of the tight junction network and actin depolymerization, eventually resulting in cell death. The lipopolysaccharide (LPS)-specific agglutinating SIgAC5 monoclonal antibody (MAb), but not monomeric IgAC5 or IgGC20 MAbs of the same specificity, achieved protective functions through combined mechanisms, including limitation of the interaction between *S. flexneri* and epithelial cells, maintenance of the tight junction seal, preservation of the cell morphology, reduction of NF-κB nuclear translocation, and inhibition of proinflammatory mediator secretion. Our results add to the understanding of the function of SIgA-mediated immune exclusion by identifying a mode of action whereby the formation of immune complexes translates into maintenance of the integrity of epithelial cells lining the mucosa. This novel mechanism of protection mediated by SIgA is important to extend the arsenal of effective strategies to fight against *S. flexneri* mucosal invasion.**

Shigella flexneri, the causative agent of bacillary dysentery, invades nonphagocytic cells through the type III secretion system (T3SS), which delivers bacterial effectors that trigger severe inflammatory reactions, eventually leading to epithelium destruction (1). Successive events, including Peyer's patch (PP) M cell-mediated entry (2), apoptosis of infected macrophages (3), and recruitment of polymorphonuclear cells that further amplify local damage (4), promote the access of *S. flexneri* to the basolateral surface of epithelial cells. Subversion of host cell architecture through the injection of effector proteins promotes the cell-to-cell propagation of infection, a process accompanied by the epithelial production of proinflammatory mediators (5). *In vitro* models using enterocyte-derived monolayers partially or not differentiated have led to the most-favored conclusion, that *S. flexneri* invades intestinal epithelial cell (IEC) monolayers exclusively from the basolateral pole (6). However, both *in vitro* and *in vivo* models have identified the effectiveness of epithelial infection from the apical brush border, arguing for an alternative site of entry for the bacterium besides PPs (7–9). Although rapid remodeling of tight junction organization by *S. flexneri* has been documented (9), the more long-term effect on IEC responsiveness is in need of investigation.

Both innate and acquired types of immune responses have been implicated in combating *S. flexneri* infection, reflecting the complexity of the protection processes (10–12). In the gastrointestinal tract, the local adaptive humoral response is essentially mediated by secretory IgA (SIgA), the main immunoglobulin found at the mucosal surface. The protective function of specific SIgA against *S. flexneri* has been described *in vivo* using rabbit ileal loops, as well as in samples from infected patients (12–17), and relies on immune exclusion, preventing epithelial damage. However, how the protective role of the antibody (Ab) is relayed to IEC wellness and, thus, its essential barrier function is not known. Recently published data demonstrated a transient suppression of the T3SS when the bacteria were incubated with the lipopolysaccharide (LPS)-specific monoclonal antibody (MAb) IgAC5 (18). This feature and the role of T3SS in initial infectious processes prompted us to examine the mechanisms by which SIgA-based protection can be mediated at the intestinal luminal surface. Polarized Caco-2 cell monolayers, serving as a mimic of the intestinal epithelium for controlling the passage of antigens and potentially harmful microorganisms, were infected with bacteria either alone or in complexes with LPS-specific SIgAC5, monomeric IgAC5, IgGC20, and the *Salmonella enterica* serovar Typhimurium-specific SIgASal4 as a nonspecific control. We found that apical exposure of Caco-2 cells to *S. flexneri* triggered progressive cytoskeletal and tight junction disorganization that favors bacterial diffusion, a chronologic process that was specifically delayed by the agglutination properties of the anti-LPS SIgAC5 MAb. SIgA-mediated neutralization of *S. flexneri* interfered with IEC responsiveness, as mapped by altered NF-κB nuclear translocation and a drop in cytokine/chemokine release. As such preventive actions did not occur in the presence of monomeric IgAC5, IgGC20, or SIgASal4, our study demonstrates the specific functions of SIgAC5 in limiting bacterial binding, maintaining epithelial cell integrity, preventing cellular dissemination of the bacterium, and reducing

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subsequent activation of proinflammatory messengers, a series of events involved in *S. flexneri* infection from the apical epithelial surface.

MATERIALS AND METHODS

Caco-2 cell culture and transepithelial electrical resistance measurements. The human colonic adenocarcinoma epithelial Caco-2 cell line (American Type Tissue Collection) was seeded on polyester Snapwell filters (diameter, 12 mm; pore size, 0.4 μm; Corning Costar) as described previously (19). The cells were grown in complete Dulbecco's modified Eagle's medium DMEM (C-DMEM) supplemented with 10% fetal calf serum (FCS; Sigma), 1% nonessential amino acids (Gibco), 1% glutamine, 10 mM HEPES (Invitrogen), 0.1% transferrin (Invitrogen), and 1% streptomycin-penicillin (Sigma). The integrity of the polarized Caco-2 cell monolayers was checked by measuring the transepithelial electrical resistance (TER) with a Millicell electrical resistance system (ERS) (Millipore). The TER values of well-differentiated monolayers ranged from 450 to 550 Ω/cm^2. A drop in TER values below 250 Ω/cm^2 is indicative of damaged cellular integrity.

Microorganisms and growth conditions. The serotype 5a, LPS-producing virulent *S. flexneri* strain M90T constitutively expressing green fluorescent protein (GFP) (20) was cultured and quantified as described previously (21).

Cell lines and protein production. Mouse IgAC5 (22) and IgGC20 (23) MAbs that are specific for *S. flexneri* serotype 5a LPS and mouse IgASal4 (24) MAb that is specific for *Salmonella* Typhimurium surface carbohydrates were cultured as described previously (25). Polymeric and monomeric forms of the IgAC5 Ab were separated by size exclusion chromatography (25). The mouse secretory component obtained from hybridoma 2H2 (26) was combined with equimolar amounts of polymeric IgA (pIgA) MAbs in phosphate-buffered saline (PBS) at room temperature (RT) to generate SIgAC5 and SIgASal4, respectively (27).

Antibody association to bacteria. Amounts of 2×10^7 bacteria were mixed with 10 μg of SIgAC5 or SIgASal4 or 2 μg of IgGC20 or monomeric IgAC5 in a final volume of 500 μl of PBS and incubated for 1 h at RT under gentle agitation. The immune complexes were washed 3 times in PBS and resuspended in plain DMEM (P-DMEM) complemented with 10 mM HEPES for analysis of MAb-mediated agglutination or bacterial growth or to infect polarized Caco-2 cell monolayers (multiplicity of infection [MOI] = 20).

The stability of immune complexes was visualized at 1 h and after overnight incubation with biotinylated goat anti-mouse Ig α chain (1/10; Cappel) or biotinylated goat anti-mouse Ig γ chain (1/50, Invitrogen), followed by cyanine 5 (Cy5)-conjugated streptavidin (1/500; GE HealthCare). Labeled immune complexes were laid onto glass slides (Thermo Scientific), fixed in 2% paraformaldehyde in PBS for 25 min at RT, mounted in Vectashield solution (Vector Laboratories), and visualized using a Zeiss LSM 710 Meta confocal microscope (Carl Zeiss, Germany) equipped with a 63× objective (imaging facility, UNI-Lausanne). Images were processed with Zeiss ZEN 2009 light software.

To measure the direct impact of the MAbs on bacterial growth, suspensions of bacteria alone or associated in immune complexes were evaluated by measuring optical density (OD; 1 OD unit at 600 nm corresponds to 1×10^8 *S. flexneri*) 3 and 6 h after the formation of immune complexes and after overnight incubation in solution at 37°C. As agglutination resulted in sedimentation of bacteria, OD was measured after resuspension. We favored this method instead of plating because agglutination by SIgAC5 might have led to a bias due to overlapping colonies emanating from immune complexes containing several bacteria.

Exposure of Caco-2 cells to bacteria. One hour before the use of polarized Caco-2 cell monolayers, C-DMEM was replaced by P-DMEM in both the apical and basolateral compartments. The apical medium was then replaced by 500 μl of bacterial suspensions (2×10^7 bacteria, MOI = 20) as such or in the form of immune complexes. TER values were measured at selected time points from the beginning of the infection onward. In selected experiments, Caco-2 cells were treated or not with 50 μg/ml gentamicin for 30 min, washed in sterile PBS, and incubated for 3 min in lysis buffer (10 mM Tris-HCl [pH 7], 0.2% Nonidet P-40, 50 mM NaCl, 2 mM EDTA [pH 8]). Lysates were seeded on LB agar plates containing 50 μg/ml of ampicillin, and CFU were determined after overnight incubation at 37°C.

Laser scanning confocal microscopy (LSCM) observation of Caco-2 cell monolayers. Infected polarized Caco-2 cell monolayers grown in Snapwell filters were washed with PBS prior to fixation overnight with 5 ml of 4% paraformaldehyde at 4°C. After washing with PBS, nonspecific binding sites on filters were blocked with PBS containing 5% FCS and 0.2% Triton X-100 (PBS-T) for 30 min at RT. All Abs were diluted in PBS-T. Filters were incubated with rabbit anti-human ZO-1 antibody (1/200, Invitrogen) for 2 h at RT and washed in PBS, followed by incubation with goat anti-rabbit IgG conjugated with Alexa Fluor 647 (1/100; Invitrogen) for 90 min at RT. When assessed, phalloidin associated with Fluoprobes 547H (1/200; Interchim) was incubated concomitantly with the secondary Ab. To visualize cells, filters were finally incubated with 100 ng/ml of 4′,6′-diamidino-2-phenylindole (DAPI) in PBS (Invitrogen) for 30 min. Filters were cut out of their holders and mounted in Vectashield solution for observation with a Zeiss LSM 710 Meta confocal microscope (Carl Zeiss) equipped with either a 10× or a 40× objective. Images were processed with Zeiss ZEN 2009 light software.

Quantification of the number of invasion foci and the overall infection area. The observation of whole filters was carried out with the 10× objective using Zeiss ZEN 2009 light software. The number of invasion foci and the sum of infected areas were automatically determined with the particle analysis tool of ImageJ software in the channel detecting GFP-expressing bacteria. When the area affected by the bacteria reached a macroscopically observable loss of the polarized monolayers, the remaining areas covered by adherent cells were determined with the differential interference contrast (DIC) channel as a substitute for the channel measuring green fluorescence.

ELISA. Human CCL3, tumor necrosis factor alpha (TNF-α), and CXCL8 in the basolateral compartment of polarized Caco-2 cell monolayers were quantitated by enzyme-linked immunosorbent assay (ELISA) with commercial kits (BD Biosciences and R&D Systems).

Analysis of NF-κB nuclear translocation. Preparation of Caco-2 cell small-scale nuclear extracts was carried out as described previously (28). Members of the NF-κB family present in the nuclei from Caco-2 cells were identified by immunoblotting (28) with rabbit antiserum directed against the p50 or p65 subunit (1/500; Santa Cruz Biotechnology) followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1/5,000; Sigma-Aldrich) and using the chemiluminescence UptiLight kit (Interchim).

Statistical analysis. The results are given as means + standard errors of the means (SEM). Two-tailed nonparametric Mann-Whitney *U*-test analysis was performed using GraphPad Prism 5 software. Differences were considered significant when P values of <0.05 were obtained.

RESULTS

Only specific anti-LPS SIgAC5 interferes with the apical infection pattern of *Shigella* in polarized Caco-2 cell monolayers. Polarized Caco-2 cell monolayers were apically exposed to virulent *S. flexneri* strain M90T alone or in complexes with specific anti-LPS SIgAC5, IgGC20, monomeric IgAC5, or irrelevant SIgASal4 MAbs. Combined measurements of TER values and numeration of bacteria present in cell lysates were used to assess the integrity of the IEC monolayers and the degree of infection, respectively. With the notable exception of incubation with M90T-SIgAC5, overnight apical infection with M90T alone or in combination with other various MAbs triggered a 70% reduction in TER values (200 Ω/cm^2), indicative of a drastic alteration of the Caco-2 monolayer (Fig. 1A). The presence of the invasion plasmid antigens in M90T

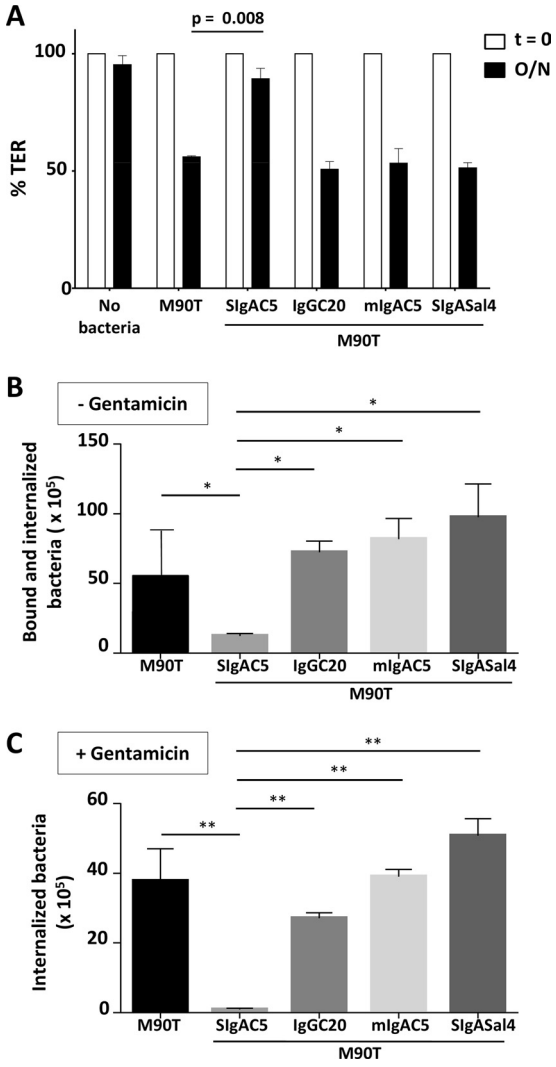

FIG 1 SIgAC5 maintains the integrity of polarized Caco-2 cell monolayers by retarding infection by virulent *S. flexneri*. (A) TER changes of intestinal Caco-2 cell monolayers exposed overnight (O/N) to *S. flexneri* M90T alone or in immune complexes with anti-LPS SIgAC5, IgGC20, or monomeric IgAC5 (mIgAC5) and irrelevant SIgASal4 as a control were monitored. Data correspond to one representative experiment ($n = 3$) for each tested condition performed in triplicates. TER of noninfected Caco-2 cell monolayers was arbitrarily set at 100%. (B, C) Adhesion/internalization of *S. flexneri* M90T alone or associated with SIgAC5, IgGC20, mIgAC5, and SIgASal4 to polarized Caco-2 cell monolayers as determined after overnight incubation in the absence (B) or presence (C) of gentamicin treatment for 30 min. Data are expressed on a per-filter basis and correspond to one representative experiment ($n = 3$) for each tested condition performed in triplicates. Mean values + SEM are shown. Statistically significant differences calculated by comparison with M90T-SIgAC5 are indicated above the columns as follows: *, $P \leq 0.05$; **, $P \leq 0.01$.

was required for IEC infection, as incubation with the avirulent *S. flexneri* mutant BS176 (29) did not affect TER (not shown). Because IgGC20 or monomeric IgAC5 with the same specificity as SIgAC5 could not compensate for the TER drop, this suggests that both the isotype and the molecular form of the Ab were essential to its protective function *in vitro*. However, antigen binding specificity was required, as SIgASal4 recognizing *Salmonella* Typhimurium surface carbohydrates did not prevent TER drop either; this further indicated that secretory component present in SIgAC5 was not involved in the preservation of Caco-2 cell monolayer integrity. Under conditions of incubation with M90T-SIgAC5, the TER was still at 70% of its original value at 24 h and dropped to 200 Ω/cm^2 after 40 h of incubation (not shown). Together, these data argue for the functional superiority of SIgA in retarding IEC infection from the apical pole and suggest that the mere presence of SIgAC5 MAb with a single specificity contributes to time-dependent, yet time-limited protection. In addition to unraveling the unique properties of a specific SIgA MAb to preserve Caco-2 cell integrity, these results demonstrate that the entry of *S. flexneri* is not restricted to the basolateral pole of IECs, in contradiction to the study of Mounier et al. (6). Various adjustments in the experimental settings, including the period of differentiation of epithelial cells on Transwell membranes, may justify such subtle differences and changes in the paradigm of the mode of infection of *S. flexneri*.

As *S. flexneri* is a nonmotile bacterium, the bacterial loads present in the cell lysates reflect the ability of the apically inoculated bacteria to adhere and infect cells. Following contact with IECs, capture of *S. flexneri* is a very rapid process (15 min) that necessitates the functional assembly of the T3SS for subsequent invasion (8). As our data (Fig. 1A) show that SIgAC5 is capable of delaying damage to polarized Caco-2 cell monolayers and that binding of pIgAC5 to *S. flexneri* results in the transient suppression of the T3SS proteins (18), we speculated that one of the functions of the MAb would be to inhibit either the binding to or/and the entry into IECs. Following overnight incubation, a marked reduction in the bacterial counts of whole-Caco-2 cell lysates was observed in the presence of SIgAC5 (Fig. 1B), whereas incubation with the other control MAbs yielded values similar to those of Caco-2 cells infected with the bacteria only (Fig. 1B). Upon treatment with gentamicin to eliminate surface-bound bacteria, the lysates of Caco-2 cells exposed to M90T-SIgAC5 (Fig. 1C) contained very low counts, suggesting that entry was efficiently inhibited, in agreement with MAb-mediated reduced attachment, a mechanism ensured by immune exclusion. Similar to TER values, these differences leveled off with increased incubation times (not shown), in support of the transient inhibition of the T3SS ensured by SIgAC5. Consistent with the lack of effect observed in the absence of antibiotic, the levels of internalized M90T in the presence of other MAbs resembled that of bacteria alone (Fig. 1C). Of note, translocated bacteria accumulated in the bottom of the wells of the basolateral compartment, preventing a potential cross-infection of the monolayers from the basolateral pole receptors for *S. flexneri* (4).

Diminished bacterial growth as a consequence of SIgAC5-mediated agglutination. In comparison with IgGC20 and monomeric IgAC5 (mIgAC5) with the same specificity, SIgAC5 proved to differentially protect the Caco-2 cell monolayer by more efficiently inhibiting *S. flexneri* attachment and entry. The avidity of polymeric IgA was shown to justify such an isotype- and molecular form-dependent difference in neutralizing *Clostridium difficile* toxin A (30). We hypothesized that in the context of a bacterium, additional features of SIgA, including its effect on bacterial growth and masking in large immune complexes, may explain its better functionality. As mentioned above, pIgAC5 suppresses the T3SS proteins and diminishes the biosynthesis of ATP (18); we therefore tested whether this can affect bacterial growth in solution as a function of time. Using the same bacterium-to-MAb ratio as in

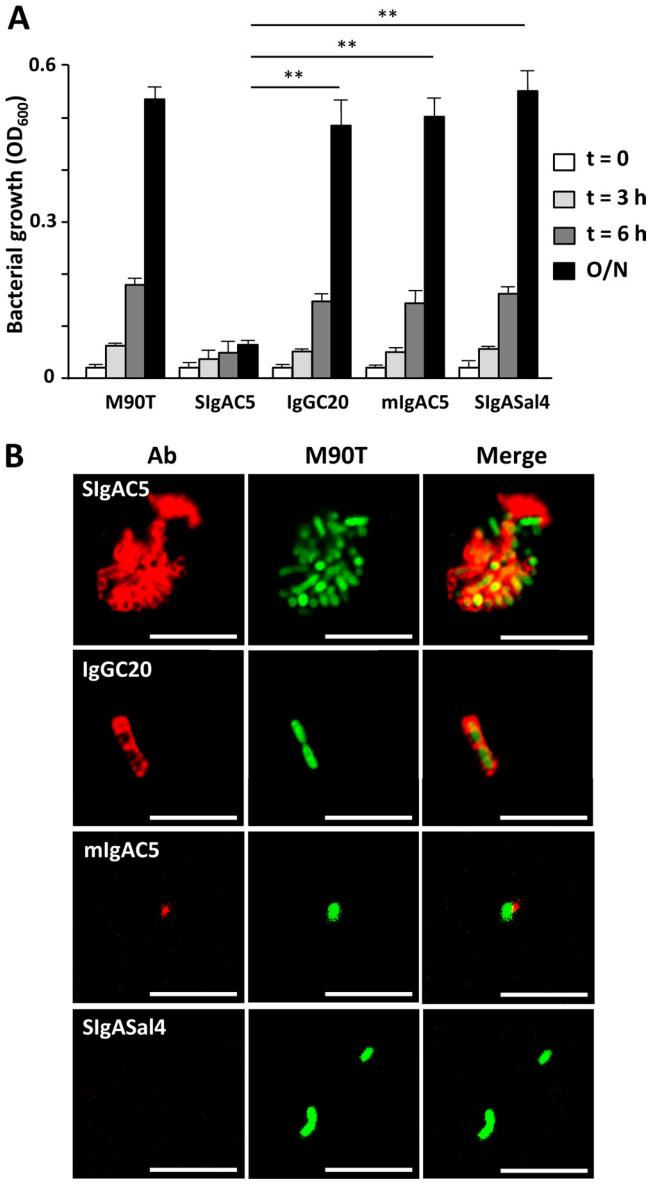

FIG 2 Bacterial agglutination occurs in the presence of LPS-specific SIgAC5 MAb only. (A) Growth of *S. flexneri* alone or in the presence of various MAbs as a function of time. Mean values + SEM are shown. Statistical differences are shown for the overnight (O/N) condition. OD_{600}, optical density at 600 nm. **, $P \leq 0.01$. (B) M90T expressing GFP was incubated with SIgAC5, IgGC20, monomeric IgAC5 (mIgAC5), and SIgASal4 stained with α chain- or γ chain-specific Abs and visualized by LSCM. Bacteria agglutinate only in the presence of SIgAC5, which contributes to the formation of immune complexes, resulting in large aggregates. Only surface coating (IgGC20 and mIgAC5) or no binding (SIgASal4) was observed with control MAbs. One representative field, obtained from 10 different observations following analysis of 3 different slides, is shown. Scale bars, 5 μm.

the experiments whose results are depicted in Figure 1, we found that a 3-h incubation with SIgAC5 reduced the bacterial counts by a factor of 1.4 compared with the results using M90T alone, while equimolar amounts of IgGC20, monomeric IgAC5, and SIgASal4 MAbs had no effect (Fig. 2A). In addition to the effect on bacterial growth, we found that aggregates formed within 1 h in the presence of tetravalent SIgAC5 only but not upon incubation with divalent specific IgGC20 or monomeric IgAC5 or nonspecific SIgASal4 of identical structure (Fig. 2B). At 6 h, only cells incubated with M90T-SIgAC5 exhibited a 2-fold reduction in bacterial counts in comparison with the counts in cells incubated with the bacteria grown alone or with the other MAbs, an effect that amplified further (up to 9-fold decrease) after overnight incubation (Fig. 2A). This strongly suggests that the impact on *S. flexneri* proliferation can account for reduced infection.

In agreement with the tetrameric valence of SIgAC5, the red-labeled MAb was present all over the opsonized lattice of M90T; in comparison, with IgGC20 and mIgAC5, the coating was limited to the surface of individual bacteria and no binding was detected for SIgASal4 (Fig. 2B). A similar pattern of immune complexes were still seen after overnight culture (data not shown), together with low levels of single bacteria, suggesting that a limited number of bacteria can escape SIgA-mediated agglutination over time. The agglutination-based capacity of anti-LPS-specific SIgAC5 to reduce binding to IECs, combined with its negative impact on *S. flexneri* growth, explains molecularly why SIgA exhibits protective functions toward sensitive IECs that are superior to those of the other MAbs tested.

SIgAC5 controls disruption of the Caco-2 cell monolayer by limiting the sites of productive infection. While the agglutination properties of SIgAC5 can justify its effect on bacteria, the beneficial impact on target IECs infected from the apical pole remains to be understood. The preserved TER and low bacterial counts (Fig. 1) strongly suggest that the neutralizing function of SIgA makes it more difficult for M90T to invade Caco-2 cells and allows the maintenance of the IEC monolayer integrity for a prolonged period. We thus analyzed quantitatively the beneficial protective role of SIgAC5 in comparison with the effects of other MAbs using LSCM images from whole Transwell filters (Fig. 3A and B). Incubation with M90T-SIgAC5 was the sole experimental condition to display a significant reduction in both the overall infected area and the number of infection foci after overnight exposure to polarized Caco-2 cell monolayers compared to the results for incubation with M90T alone and M90T in complex with the other MAbs tested. Upon analysis of LSCM images reflecting the representative pattern of each experimental condition, we found that surface-bound bacteria were neutralized by the SIgAC5 MAb only, while unlimited spreading occurred all over the monolayers in the other scenarios (Fig. 3C). SIgAC5 also drastically limited the expansion of infected foci and the development of large areas devoid of cells, as was observed with M90T alone or in complex with the other MAbs (Fig. 3C). Taken together, these results suggest that the agglutinating features of SIgAC5 restrict bacterial dissemination among neighbor cells (Fig. 3C), further supporting the concept that SIgA Abs elicited upon primary M90T infection are important in protecting against IEC reinfection and that this occurs by ensuring morphological integrity.

SIgAC5 delays destruction of the tight junction network and actin-based cell architecture. The data shown in Figure 3C suggest that IECs infected with *S. flexneri* undergo important structural changes, as revealed by the disappearance of the tight junction network and formation of areas devoid of cells. Within hours postinfection, the architecture of actin fibers changed drastically only in the periphery of the sites of interaction with M90T, supporting the idea that rearrangement of the cytoskeleton is induced by virulence factors expressed by the invading bacteria (Fig. 4A, arrowheads). Actin remodeling would aim at preventing excessive

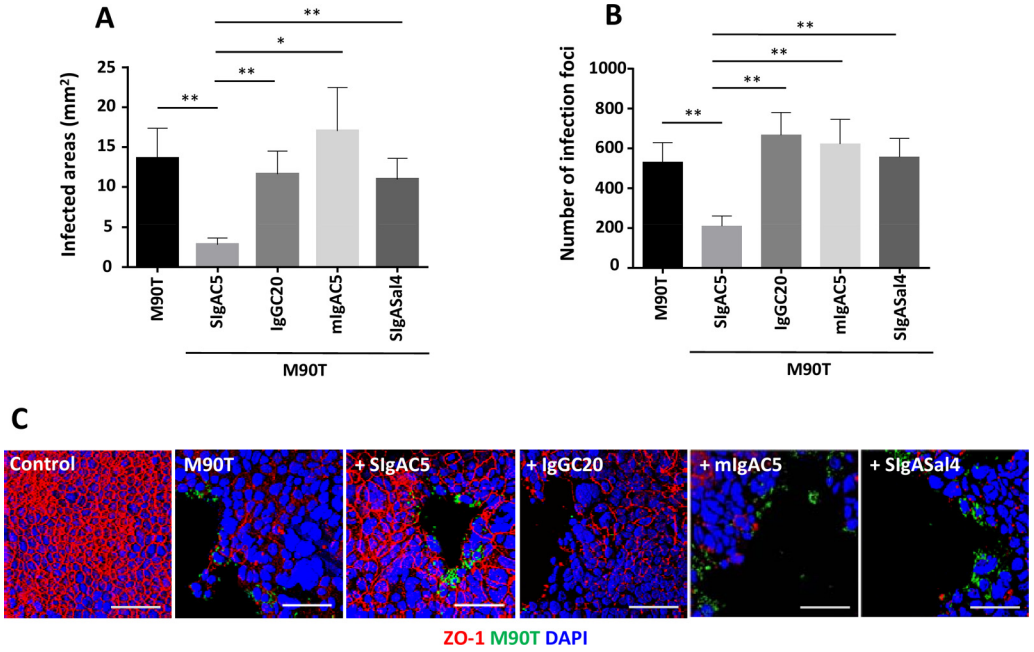

FIG 3 SIgA maintains Caco-2 cell monolayer integrity and limits M90T cellular dissemination. Damage inflicted on Caco-2 cell monolayers by apical addition of M90T alone or combined with various MAbs after overnight incubation was determined by measuring the sum of infected areas (A) and the number of infection foci (B) from LSCM images using ImageJ software. Data are expressed on a per-filter basis. Mean values + SEM are shown; $n = 3$ experiments carried out in triplicates. Statistically significant differences calculated by comparison with M90T-SIgAC5 are indicated above the columns as follows: **, $P \leq 0.01$; *, $P \leq 0.05$. (C) LSCM 3-dimensional reconstructed images (snapshot) of Caco-2 cell monolayers exposed overnight to M90T alone or in combination with various MAbs. Limited dissemination of bacteria (green) and maintenance of tight junctions stabilizing the monolayer (ZO-1 red labeling) was visualized with SIgAC5 only, while tight junction disappearance induced by uncontrolled infection is observed for all other experimental conditions. Caco-2 cell nuclei were stained with DAPI (blue). One representative field obtained from the observation of whole Transwell filters recovered from 3 experiments performed in triplicates is shown. Scale bars, 50 μm.

cellular damage during intracellular bacterial proliferation, allowing transient maintenance of local cohesion (ZO-1 distribution is preserved), a stratagem most likely achieved by the recently described OspE virulence factor (31, 32). The loss of the ZO-1 signal after overnight incubation reflects the beginning of the destruction of intercellular junctions (Fig. 4B) and is accompanied by the disappearance of the cell architecture, as mapped by the complete extinction of phalloidin detection. The *S. flexneri*-induced loss of the tight junction network and actin fiber depolymerization suggest a novel mode of invasion from the apical surface which promotes rapid bacterial propagation and dissemination within the monolayers, a process that may synergize with bacteria invading from the basolateral pole after M cell-mediated entry.

In sharp contrast, the aggregation mediated by SIgAC5 re-

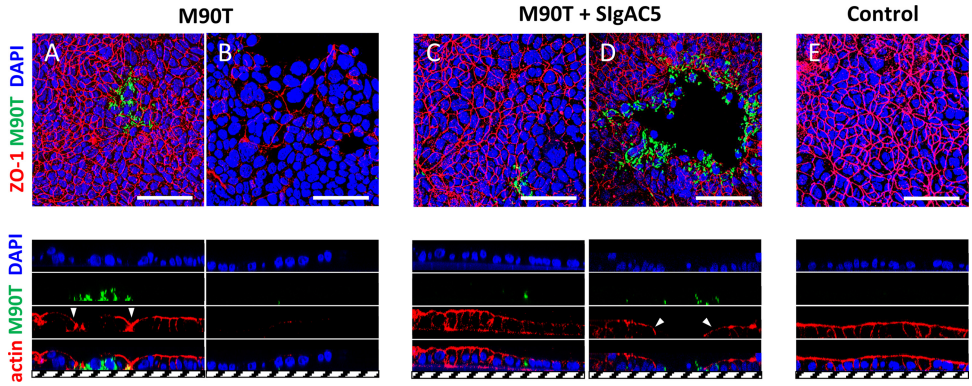

FIG 4 SIgA-mediated protection delays the disruption of tight junctions and depolymerization of actin fibers. LSCM 3-dimensional reconstructed images (snapshot) of Caco-2 cell monolayers exposed to M90T alone or in combination with SIgAC5 are shown; views are from the top (top panels) and along the ZX plan (bottom panels). (A) Invasive M90T (green) located intracellularly triggers actin remodeling at 10 h, as tracked by phalloidin staining (red, bottom panels). (B) Destruction of tight junctions (red, top panel) occurs 6 h later. (C) Limited infection in the presence of SIgAC5 prevents destruction of tight junctions and organized actin fibers. (D) Neutralization by the Ab results in delayed damage after overnight exposure. (E) Noninfected Caco-2 cell monolayers are depicted for comparison. The basal side of the Caco-2 cell monolayer is displayed as hatching on the bottom images. Sites of extensive actin remodeling are pinpointed by white arrowheads. Images are one example of 90 observed among 3 filters prepared from 3 independent experiments performed in triplicates. Scale bars, 50 μm.

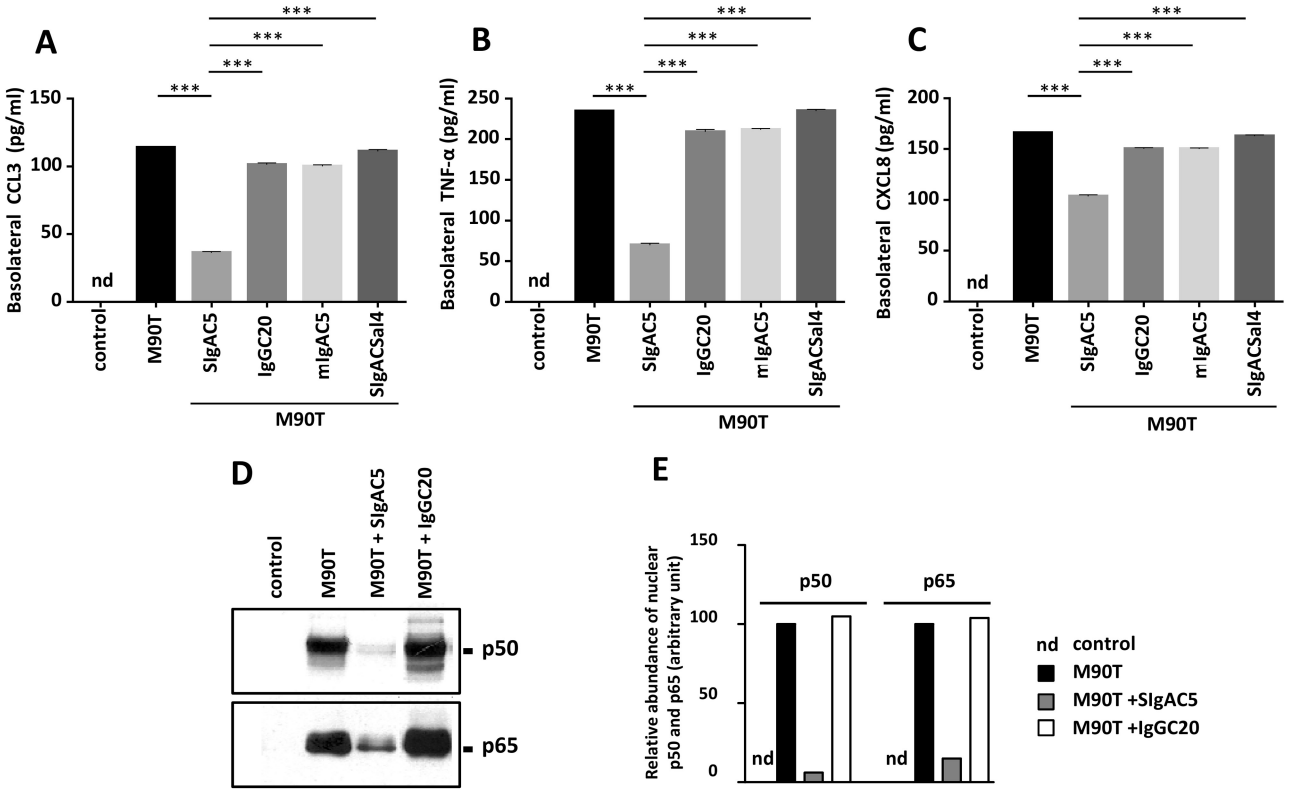

FIG 5 Anti-inflammatory properties of SIgA reduce secretion of proinflammatory mediators by polarized Caco-2 cell monolayers through inhibition of NF-κB nuclear translocation. Production of CCL3 (A), TNF-α (B), and CXCL8 (C) was measured in the basolateral compartment of Caco-2 cell monolayers incubated under various conditions after overnight incubation. Mean values + SEM are shown; $n = 3$ experiments in triplicate. ***, $P \leq 0.001$. (D) Immunoblotting of the NF-κB subunits p50 and p65/RelA in the nuclear extracts from Caco-2 cell monolayers apically incubated overnight with M90T alone or in complex with LPS-specific SIgAC5 and IgGC20 as a control. Panels are representative of one individual experiment performed in triplicate ($n = 3$). (E) Densitometric analysis of immunoblots described for panel D, exposed for optimal times to avoid saturation of the photographic film. The intensity of the signal reached with M90T alone was fixed at 100%. nd, not detectable.

sulted in punctate surface binding and prevented bacterial spreading, thus ensuring limited damage to a few targeted epithelial cells (Fig. 4C and D). Strikingly, outside the infected foci and despite a slight decrease in the detection of pericellular ZO-1 and organized actin fibers, exposure of Caco-2 cells to M90T-SIgAC5 overnight resulted in patterns similar to those in uninfected filters (Fig. 4D and E). Similar control experiments performed with M90T-IgGC20 revealed infection patterns similar to those observed for M90T alone (not shown).

SIgAC5 represses CCL3, TNF-α, and CXCL8 secretion by IECs via blocking of NF-κB nuclear translocation. We next hypothesized that the neutralizing ability of SIgAC5 would result in moderate apical sensing of the M90T strain by polarized Caco-2 cell monolayers. The production of CCL3 (macrophage inflammatory protein-1α), TNF-α, and CXCL8 (interleukin-8 [IL-8]) in the basolateral compartment, as well as the nuclear translocation of NF-κB, known to regulate the expression of the three mediators, were examined as markers of the Caco-2 cell proinflammatory response (5, 11, 33). SIgAC5 interacting with the M90T strain significantly reduced the production of CCL3 (≈70%), TNF-α (≈70%), and CXCL8 (≈35%) by Caco-2 cells compared with the levels obtained by incubation with M90T alone or in complex with the other MAbs tested (Fig. 5A, B, and C). This reflects in IECs the situation observed in the rabbit model, in which the expression of proinflammatory mediators in the Peyer's patch tissue is quenched when *S. flexneri* is administered as a complex with SIgAC5 (13). High nuclear translocation of the transcription factor NF-κB subunits p50 and p65 occurred after incubation with M90T alone or in complex with specific IgGC20 (Fig. 5D and E), whereas the exposure of Caco-2 cells to SIgAC5-based immune complexes led to a marked drop in the nuclear detection of either NF-κB subunit (Fig. 5D and E). The data reveal the prominent role of neutralizing extracellular SIgA in controlling the onset of cellular proinflammatory responses (7), a feature that contributes to maintaining the physical integrity of the epithelial barrier.

DISCUSSION

The functions of SIgA at mucosal surfaces are manifold, extending from transport of immune complexes across PPs, control of inflammatory circuits, intracellular neutralization of invading pathogens, and regulation of the microbiota to classical/paradigm immune exclusion (34). Despite the *in vivo* and *in vitro* demonstration of the importance of SIgA in the latter process, it remains unclear by which underlying mechanisms extracellular SIgA capable of preventing invasion can maintain short-range epithelial integrity. The results of the present study demonstrate that agglutinating SIgA precludes contact of the enteropathogen *S. flexneri* with target IECs, resulting in maintenance of tight junctions and

cell morphology and silencing of cellular proinflammatory pathways. Remarkably, this occurs in the absence of other immune partners usually involved in combating *S. flexneri* infection. Furthermore, the lack of mucus, known to contribute to repelling bacteria (35) and anchoring SIgA for improved functionality (36), does not negate the crucial role of SIgA in ensuring prolonged preservation of the polarized IEC monolayer. Similarly to SIgA-controlled entry of immune complexes through PPs, our data pave the way to exploring whether selective entry into IECs via CD71 (37) may contribute to the modulation of immune reactions, for example, allergy or gut inflammation.

It has been shown that the association of pIgAC5 with *S. flexneri* de-energized the T3SS by affecting the proton motive force and reducing cellular levels of ATP (18). Temporary incapacity of the bacterium to invade epithelial cells was suggested yet not experimentally tackled. Our results indicate that perturbation of the bacterial bioenergetics by SIgAC5-mediated agglutination leads to decreased growth rate, and this translates into delayed invasion of IECs compared to the time to invade their uncoated counterparts. Inhibition of other bacterial functions by IgA, i.e., motility, ultimately affecting bacterial entry into IECs has been reported for *Salmonella enterica* (38). Interestingly, masking of LPS and adhesins by Ab coating is not sufficient to disarm *S. flexneri*, as IgGC20 does not interfere with IEC invasion; this suggests that the lattice formed by bound SIgA may trigger mechanical constraints on the bacterial wall (39), leading to metabolic alterations not seen with monomeric IgA or IgG. This is reflected by the unique property of SIgAC5 to significantly lower the levels of CCL-3, TNF-α, and CXCL8 secreted by IECs when bound to *S. flexneri* in immune complexes. Reduced epithelial secretions due to SIgA-neutralized *S. flexneri* would be indicative of the ongoing immune response, with downregulation of proinflammatory signaling (13).

Another finding of our work resides in the observation that an LPS-specific SIgA MAb, but not IgA or IgG MAbs of the same specificity, inhibits the sequential lesions induced by luminal application of the virulent *S. flexneri* M90T strain in an intestinal epithelium model. The experimental setting was designed to study the effect of M90T-triggered damage after overnight infection, well beyond the time course usually accessible in *in vivo* models (13, 22). Bacteria were initially found to infect a limited number of polarized cells, leading to preferential targeting of the tight junction's seal; such a feature has been described for *S. flexneri* serotype 2 and also for other pathogens, such as enterohemorrhagic *Escherichia coli* and *Salmonella* strains (8, 9, 39), yet at very high MOI that do not reflect the low doses sufficient to infect the human gut. Over time, the progressive overwhelming proliferation of *S. flexneri* induced irreversible damage to the cell architecture, as reflected by the complete depolymerization of actin fibers. Loss of epithelial integrity is a well-accepted consequence of bacillary dysentery (40), and it appears to be mimicked in the *in vitro* model after bacterial exposure limited to the apical epithelial cell surface.

In vitro dissection turned out to be appropriate to evaluate the multilevel neutralizing properties of SIgAC5 directed against LPS from *S. flexneri*, as reflected in the delay of infection via mechanisms that included blocking of interaction with IEC monolayers, the reduction of bacterial growth and proliferation inside polarized Caco-2 cells, maintenance of the tight junction network, slowing down of actin fiber depolymerization, and interference with the activation of proinflammatory gene products, a sum of properties that can be assigned to the agglutinating characteristics of the SIgAC5 Ab isotype. Indeed, the combination of M90T with an LPS-specific IgGC20 and mIgAC5 MAb resulted in the same pattern of sequential IEC destruction as observed with M90T alone. However, following parenteral vaccination with O-specific polysaccharide 2a, transudating polyclonal IgG was found to protect the vaccinees (41), suggesting that multiple mechanisms staggered over time may be involved. In the absence of other levels of immune protection, this work sheds light on the functional role of luminal SIgA in interfering with infection by *S. flexneri* from the apical surface. This also suggests that by interfering with the very first and destructive steps of infection, LPS-specific SIgA must be considered an asset in the battery of molecular and cellular agents required in immunity against *S. flexneri*.

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REFERENCES

1. **Sansonetti PJ.** 2006. The bacterial weaponry: lessons from Shigella. Ann. N. Y. Acad. Sci. **1072:**307–312.
2. **Wassef JS, Keren DF, Mailloux JL.** 1989. Role of M cells in initial antigen uptake and in ulcer formation in the rabbit intestinal loop model of shigellosis. Infect. Immun. **57:**858–863.
3. **Zychlinsky A, Prevost MC, Sansonetti PJ.** 1992. Shigella flexneri induces apoptosis in infected macrophages. Nature **358:**167–169.
4. **Phalipon A, Sansonetti PJ.** 1999. Microbial-host interactions at mucosal sites. Host response to pathogenic bacteria at mucosal sites. Curr. Top. Microbiol. Immunol. **236:**163–189.
5. **Fernandez MI, Pedron T, Tournebize R, Olivo-Marin JC, Sansonetti PJ, Phalipon A.** 2003. Anti-inflammatory role for intracellular dimeric immunoglobulin A by neutralization of lipopolysaccharide in epithelial cells. Immunity **18:**739–749.
6. **Mounier J, Vasselon T, Hellio R, Lesourd M, Sansonetti PJ.** 1992. Shigella flexneri enters human colonic Caco-2 epithelial cells through the basolateral pole. Infect. Immun. **60:**237–248.
7. **Perdomo OJ, Cavaillon JM, Huerre M, Ohayon H, Gounon P, Sansonetti PJ.** 1994. Acute inflammation causes epithelial invasion and mucosal destruction in experimental shigellosis. J. Exp. Med. **180:**1307–1319.
8. **Romero S, Grompone G, Carayol N, Mounier J, Guadagnini S, Prevost MC, Sansonetti PJ, Van Nhieu GT.** 2011. ATP-mediated Erk1/2 activation stimulates bacterial capture by filopodia, which precedes Shigella invasion of epithelial cells. Cell Host Microbe **9:**508–519.
9. **Sakaguchi T, Kohler H, Gu X, McCormick BA, Reinecker HC.** 2002. Shigella flexneri regulates tight junction-associated proteins in human intestinal epithelial cells. Cell. Microbiol. **4:**367–381.
10. **Le-Barillec K, Magalhaes JG, Corcuff E, Thuizat A, Sansonetti PJ, Phalipon A, Di Santo JP.** 2005. Roles for T and NK cells in the innate immune response to Shigella flexneri. J. Immunol. **175:**1735–1740.
11. **Sansonetti PJ, Arondel J, Huerre M, Harada A, Matsushima K.** 1999. Interleukin-8 controls bacterial transepithelial translocation at the cost of epithelial destruction in experimental shigellosis. Infect. Immun. **67:**1471–1480.
12. **Schultsz C, Qadri F, Hossain SA, Ahmed F, Ciznar I.** 1992. Shigella-specific IgA in saliva of children with bacillary dysentery. FEMS Microbiol. Immunol. **4:**65–72.
13. **Boullier S, Tanguy M, Kadaoui KA, Caubet C, Sansonetti P, Corthésy B, Phalipon A.** 2009. Secretory IgA-mediated neutralization of Shigella flexneri prevents intestinal tissue destruction by down-regulating inflammatory circuits. J. Immunol. **183:**5879–5885.
14. **Cohen D, Block C, Green MS, Lowell G, Ofek I.** 1989. Immunoglobulin M, A, and G antibody response to lipopolysaccharide O antigen in symptomatic and asymptomatic Shigella infections. J. Clin. Microbiol. **27:**162–167.
15. **Islam D, Veress B, Bardhan PK, Lindberg AA, Christensson B.** 1997. Quantitative assessment of IgG and IgA subclass producing cells in rectal mucosa during shigellosis. J. Clin. Pathol. **50:**513–520.

16. **Phalipon A, Michetti P, Kaufmann M, Cavaillon JM, Huerre M, Kraehenbuhl JP, Sansonetti PJ.** 1994. Protection against invasion of the mouse pulmonary epithelium by a monoclonal IgA directed against Shigella flexneri lipopolysaccharide. Ann. N. Y. Acad. Sci. **730:**356–358.
17. **Rasolofo-Razanamparany V, Cassel-Beraud AM, Roux J, Sansonetti PJ, Phalipon A.** 2001. Predominance of serotype-specific mucosal antibody response in Shigella flexneri-infected humans living in an area of endemicity. Infect. Immun. **69:**5230–5234.
18. **Forbes SJ, Bumpus T, McCarthy EA, Corthésy B, Mantis NJ.** 2011. Transient suppression of Shigella flexneri type 3 secretion by a protective O-antigen-specific monoclonal IgA. mBio **2:**00042–11. doi:10.1128/mBio.00042-11.
19. **Thierry AC, Bernasconi E, Mercenier A, Corthésy B.** 2009. Conditioned polarized Caco-2 cell monolayers allow to discriminate for the ability of gut-derived microorganisms to modulate permeability and antigen-induced basophil degranulation. Clin. Exp. Allergy **39:**527–536.
20. **Rathman M, Jouirhi N, Allaoui A, Sansonetti P, Parsot C, Tran Van Nhieu G.** 2000. The development of a FACS-based strategy for the isolation of Shigella flexneri mutants that are deficient in intercellular spread. Mol. Microbiol. **35:**974–990.
21. **Kadaoui KA, Corthésy B.** 2007. Secretory IgA mediates bacterial translocation to dendritic cells in mouse Peyer's patches with restriction to mucosal compartment. J. Immunol. **179:**7751–7757.
22. **Phalipon A, Kaufmann M, Michetti P, Cavaillon JM, Huerre M, Sansonetti P, Kraehenbuhl JP.** 1995. Monoclonal immunoglobulin A antibody directed against serotype-specific epitope of Shigella flexneri lipopolysaccharide protects against murine experimental shigellosis. J. Exp. Med. **182:**769–778.
23. **Phalipon A, Costachel C, Grandjean C, Thuizat A, Guerreiro C, Tanguy M, Nato F, Vulliez-Le Normand B, Bélot F, Wright K, Marcel-Peyre V, Sansonetti PJ, Mulard LA.** 2006. Characterization of functional oligosaccharide mimics of the Shigella flexneri serotype 2a O-antigen: implications for the development of a chemically defined glycoconjugate vaccine. J. Immunol. **176:**1686–1694.
24. **Michetti P, Mahan MJ, Slauch JM, Mekalanos JJ, Neutra MR.** 1992. Monoclonal secretory immunoglobulin A protects mice against oral challenge with the invasive pathogen Salmonella typhimurium. Infect. Immun. **60:**1786–1792.
25. **Favre LI, Spertini F, Corthésy B.** 2003. Simplified procedure to recover recombinant antigenized secretory IgA to be used as a vaccine vector. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. **786:**143–151.
26. **Crottet P, Cottet S, Corthésy B.** 1999. Expression, purification and biochemical characterization of recombinant murine secretory component: a novel tool in mucosal immunology. Biochem. J. **341:**299–306.
27. **Crottet P, Corthésy B.** 1998. Secretory component delays the conversion of secretory IgA into antigen-binding competent F(ab′)2: a possible implication for mucosal defense. J. Immunol. **161:**5445–5453.
28. **Cottet S, Corthésy-Theulaz I, Spertini F, Corthésy B.** 2002. Microaerophilic conditions permit to mimic in vitro events occurring during in vivo Helicobacter pylori infection and to identify Rho/Ras-associated proteins in cellular signaling. J. Biol. Chem. **277:**33978–33986.
29. **Sansonetti PJ, Kopecko DJ, Formal SB.** 1982. Involvement of a plasmid in the invasive ability of Shigella flexneri. Infect. Immun. **35:**852–860.
30. **Stubbe H, Berdoz J, Kraehenbuhl JP, Corthésy B.** 2000. Polymeric IgA is superior to monomeric IgA and IgG carrying the same variable domain in preventing Clostridium difficile toxin A damaging of T84 monolayers. J. Immunol. **164:**1952–1960.
31. **Kim M, Ogawa M, Fujita Y, Yoshikawa Y, Nagai T, Koyama T, Nagai S, Lange A, Fässler R, Sasakawa C.** 2009. Bacteria hijack integrin-linked kinase to stabilize focal adhesions and block cell detachment. Nature **459:**578–582.
32. **Kim M, Ogawa M, Mimuro H, Sasakawa C.** 2010. Reinforcement of epithelial cell adhesion to basement membrane by a bacterial pathogen as a new infectious stratagem. Virulence **1:**52–55.
33. **Pedron T, Thibault C, Sansonetti PJ.** 2003. The invasive phenotype of Shigella flexneri directs a distinct gene expression pattern in the human intestinal epithelial cell line Caco-2. J. Biol. Chem. **278:**33878–33886.
34. **Mantis NJ, Rol N, Corthésy B.** 2011. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. Mucosal Immunol. **4:**603–611.
35. **Johansson ME, Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC.** 2008. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proc. Natl. Acad. Sci. U. S. A. **105:**15064–15069.
36. **Corthésy B.** 2010. Role of secretory immunoglobulin A and secretory component in the protection of mucosal surfaces. Future Microbiol. **5:**817–829.
37. **Lebreton C, Ménard S, Abed J, Moura IC, Coppo R, Dugave C, Monteiro RC, Fricot A, Traore MG, Griffin M, Cellier C, Malamut G, Cerf-Bensussan N, Heyman M.** 2012. Interactions among secretory immunoglobulin A, CD71, and transglutaminase-2 affect permeability of intestinal epithelial cells to gliadin peptides. Gastroenterology **143:**698–707.
38. **Forbes SJ, Eschmann M, Mantis NJ.** 2008. Inhibition of Salmonella enterica serovar Typhimurium motility and entry into epithelial cells by a protective antilipopolysaccharide monoclonal immunoglobulin A antibody. Infect. Immun. **76:**4137–4144.
39. **Horejs C, Ristl R, Tscheliessnig R, Sleytr UB, Pum D.** 2011. Single-molecule force spectroscopy reveals the individual mechanical unfolding pathways of a surface layer protein. J. Biol. Chem. **286:**27416–27424.
40. **Guttman JA, Finlay BB.** 2009. Tight junctions as targets of infectious agents. Biochim. Biophys. Acta **1788:**832–841.
41. **Phalipon A, Tanguy M, Grandjean C, Guerreiro C, Bélot F, Cohen D, Sansonetti PJ, Mulard LA.** 2009. A synthetic carbohydrate-protein conjugate vaccine candidate against Shigella flexneri 2a infection. J. Immunol. **182:**2241–2247.

References

1. Betton GR. A review of the toxicology and pathology of the gastrointestinal tract. *Cell biology and toxicology* 2013; **29**(5)**:** 321-338.

2. Reed KK, Wickham R. Review of the gastrointestinal tract: from macro to micro. *Seminars in oncology nursing* 2009; **25**(1)**:** 3-14.

3. DeSesso JM, Jacobson CF. Anatomical and physiological parameters affecting gastrointestinal absorption in humans and rats. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 2001; **39(**3)**:** 209-228.

4. van der Flier LG, Clevers H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. *Annual review of physiology* 2009; **71:** 241-260.

5. Li P, Lin JE, Marszlowicz GP, Valentino MA, Chang C, Schulz S *et al*. GCC signaling in colorectal cancer: Is colorectal cancer a paracrine deficiency syndrome? *Drug news & perspectives* 2009; **22**(6)**:** 313-318.

6. Bevins CL, Salzman NH. Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis. *Nature reviews Microbiology* 2011; **9**(5)**:** 356-368.

7. Kim YS, Ho SB. Intestinal goblet cells and mucins in health and disease: recent insights and progress. *Current gastroenterology reports* 2010; **12**(5)**:** 319-330.

8. Moran GW, Leslie FC, Levison SE, Worthington J, McLaughlin JT. Enteroendocrine cells: neglected players in gastrointestinal disorders? *Therapeutic advances in gastroenterology* 2008; **1**(1)**:** 51-60.

9. Henderson P, van Limbergen JE, Schwarze J, Wilson DC. Function of the intestinal epithelium and its dysregulation in inflammatory bowel disease. *Inflammatory bowel diseases* 2011; **17**(1)**:** 382-395.

10. Snoeck V, Goddeeris B, Cox E. The role of enterocytes in the intestinal barrier function and antigen uptake. *Microbes and infection / Institut Pasteur* 2005; **7**(7-8)**:** 997-1004.

11. Bach SP, Renehan AG, Potten CS. Stem cells: the intestinal stem cell as a paradigm. *Carcinogenesis* 2000; **21**(3)**:** 469-476.

12. Barker N, van de Wetering M, Clevers H. The intestinal stem cell. *Genes & development* 2008; **22**(14)**:** 1856-1864.

13. Ulluwishewa D, Anderson RC, McNabb WC, Moughan PJ, Wells JM, Roy NC. Regulation of tight junction permeability by intestinal bacteria and dietary components. *The Journal of nutrition* 2011; **141**(5)**:** 769-776.

14. Furuse M. Molecular basis of the core structure of tight junctions. *Cold Spring Harbor perspectives in biology* 2010; **2**(1)**:** a002907.

15. Harris TJ, Tepass U. Adherens junctions: from molecules to morphogenesis. *Nature reviews Molecular cell biology* 2010; **11**(7)**:** 502-514.

16. Niessen CM. Tight junctions/adherens junctions: basic structure and function. *The Journal of investigative dermatology* 2007; **127**(11)**:** 2525-2532.

17. Desai BV, Harmon RM, Green KJ. Desmosomes at a glance. *Journal of cell science* 2009; **122**(Pt 24)**:** 4401-4407.

18. Corthésy B. Roundtrip ticket for secretory IgA: role in mucosal homeostasis? *Journal of immunology* 2007; **178**(1)**:** 27-32.

19. Corthésy B. Secretory immunoglobulin A: well beyond immune exclusion at mucosal surfaces. *Immunopharmacology and immunotoxicology* 2009; **31**(2)**:** 174-179.

20. Pearson C, Uhlig HH, Powrie F. Lymphoid microenvironments and innate lymphoid cells in the gut. *Trends in immunology* 2012; **33**(6)**:** 289-296.

21. Fagarasan S, Honjo T. Intestinal IgA synthesis: regulation of front-line body defences. *Nature reviews Immunology* 2003; **3**(1)**:** 63-72.

22. von Andrian UH, Mempel TR. Homing and cellular traffic in lymph nodes. *Nature reviews Immunology* 2003; **3**(11)**:** 867-878.

23. Mowat AM, Viney JL. The anatomical basis of intestinal immunity. *Immunological reviews* 1997; **156:** 145-166.

24. Azzali G. Structure, lymphatic vascularization and lymphocyte migration in mucosa-associated lymphoid tissue. *Immunological reviews* 2003; **195:** 178-189.

25. Makala LH, Suzuki N, Nagasawa H. Peyer's patches: organized lymphoid structures for the induction of mucosal immune responses in the intestine. *Pathobiology : journal of immunopathology, molecular and cellular biology* 2002; **70**(2)**:** 55-68.

26. Jung C, Hugot JP, Barreau F. Peyer's Patches: The Immune Sensors of the Intestine. *International journal of inflammation* 2010; **2010:** 823710.

27. Takahashi I, Nochi T, Yuki Y, Kiyono H. New horizon of mucosal immunity and vaccines. *Current opinion in immunology* 2009; **21**(3)**:** 352-358.

28. Ouyang W, Kolls JK, Zheng Y. The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity* 2008; **28**(4)**:** 454-467.

29. Pabst O. New concepts in the generation and functions of IgA. *Nature reviews Immunology* 2012; **12**(12)**:** 821-832.

30. Corthésy B. Role of secretory immunoglobulin A and secretory component in the protection of mucosal surfaces. *Future microbiology* 2010; **5**(5)**:** 817-829.

31. Woof JM, Russell MW. Structure and function relationships in IgA. *Mucosal immunology* 2011; **4**(6)**:** 590-597.

32. Corthésy B. Recombinant secretory immunoglobulin A in passive immunotherapy: linking immunology and biotechnology. *Current pharmaceutical biotechnology* 2003; **4**(1)**:** 51-67.

33. Otten MA, van Egmond M. The Fc receptor for IgA (FcalphaRI, CD89). *Immunology letters* 2004; **92**(1-2)**:** 23-31.

34. Mantis NJ, Rol N, Corthésy B. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal immunology* 2011; **4**(6)**:** 603-611.

35. Brandtzaeg P. Update on mucosal immunoglobulin A in gastrointestinal disease. *Current opinion in gastroenterology* 2010; **26**(6)**:** 554-563.

36. Monteiro RC. The role of IgA and IgA Fc receptors as anti-inflammatory agents. *Journal of clinical immunology* 2010; **30 Suppl 1:** S61-64.

37. Woof JM, Mestecky J. Mucosal immunoglobulins. *Immunological reviews* 2005; **206:** 64-82.

38. Mestecky J, Moro I, Underdown BJ. Mucosal immunoglobulins. *Mucosal Immunology (eds)* 1999; **Academic Press, San Diego,:** 133-352.

39. Mattu TS, Pleass RJ, Willis AC, Kilian M, Wormald MR, Lellouch AC *et al.* The glycosylation and structure of human serum IgA1, Fab, and Fc regions and the role of N-glycosylation on Fcalpha receptor interactions. *The Journal of biological chemistry* 1998; **273**(4)**:** 2260-2272.

40. Royle L, Roos A, Harvey DJ, Wormald MR, van Gijlswijk-Janssen D, Redwan el RM *et al.* Secretory IgA N- and O-glycans provide a link between the innate and adaptive immune systems. *The Journal of biological chemistry* 2003; **278**(22)**:** 20140-20153.

41. Johansen FE, Braathen R, Brandtzaeg P. Role of J chain in secretory immunoglobulin formation. *Scandinavian journal of immunology* 2000; **52**(3)**:** 240-248.

42. Brandtzaeg P. Secretory IgA: Designed for Anti-Microbial Defense. *Frontiers in immunology* 2013; **4:** 222.

43. Hughes GJ, Reason AJ, Savoy L, Jaton J, Frutiger-Hughes S. Carbohydrate moieties in human secretory component. *Biochimica et biophysica acta* 1999; **1434**(1)**:** 86-93.

44. Fallgreen-Gebauer E, Gebauer W, Bastian A, Kratzin HD, Eiffert H, Zimmermann B *et al.* The covalent linkage of secretory component to IgA. Structure of sIgA. *Biological chemistry Hoppe-Seyler* 1993; **374**(11)**:** 1023-1028.

45. Crottet P, Corthésy B. Mapping the interaction between murine IgA and murine secretory component carrying epitope substitutions reveals a role of domains II and III in covalent binding to IgA. *The Journal of biological chemistry* 1999; **274**(44)**:** 31456-31462.

46. Corthésy B. Multi-faceted functions of secretory IgA at mucosal surfaces. *Frontiers in immunology* 2013; **4:** 185.

47. Apter FM, Lencer WI, Finkelstein RA, Mekalanos JJ, Neutra MR. Monoclonal immunoglobulin A antibodies directed against cholera toxin prevent the toxin-induced chloride secretory response and block toxin binding to intestinal epithelial cells in vitro. *Infection and immunity* 1993; **61**(12)**:** 5271-5278.

48. Mantis NJ, McGuinness CR, Sonuyi O, Edwards G, Farrant SA. Immunoglobulin A antibodies against ricin A and B subunits protect epithelial cells from ricin intoxication. *Infection and immunity* 2006; **74**(6)**:** 3455-3462.

49. Stubbe H, Berdoz J, Kraehenbuhl JP, Corthésy B. Polymeric IgA is superior to monomeric IgA and IgG carrying the same variable domain in preventing Clostridium difficile toxin A damaging of T84 monolayers. *Journal of immunology* 2000; **164**(4)**:** 1952-1960.

50. Michetti P, Porta N, Mahan MJ, Slauch JM, Mekalanos JJ, Blum AL *et al.* Monoclonal immunoglobulin A prevents adherence and invasion of polarized epithelial cell monolayers by Salmonella typhimurium. *Gastroenterology* 1994; **107**(4)**:** 915-923.

51. Cravioto A, Tello A, Villafan H, Ruiz J, del Vedovo S, Neeser JR. Inhibition of localized adhesion of enteropathogenic Escherichia coli to HEp-2 cells by immunoglobulin and oligosaccharide fractions of human colostrum and breast milk. *The Journal of infectious diseases* 1991; **163**(6)**:** 1247-1255.

52. Carbonare CB, Carbonare SB, Carneiro-Sampaio MM. Secretory immunoglobulin A obtained from pooled human colostrum and milk for oral passive immunization. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology* 2005; **16**(7)**:** 574-581.

53. Apter FM, Michetti P, Winner LS, 3rd, Mack JA, Mekalanos JJ, Neutra MR. Analysis of the roles of antilipopolysaccharide and anti-cholera toxin immunoglobulin A (IgA) antibodies in protection against Vibrio cholerae and cholera toxin by use of monoclonal IgA antibodies in vivo. *Infection and immunity* 1993; **61**(12)**:** 5279-5285.

54. Czinn SJ, Cai A, Nedrud JG. Protection of germ-free mice from infection by Helicobacter felis after active oral or passive IgA immunization. *Vaccine* 1993; **11**(6)**:** 637-642.

55. Phalipon A, Kaufmann M, Michetti P, Cavaillon JM, Huerre M, Sansonetti P *et al.* Monoclonal immunoglobulin A antibody directed against serotype-specific epitope of Shigella flexneri lipopolysaccharide protects against murine experimental shigellosis. *The Journal of experimental medicine* 1995; **182**(3)**:** 769-778.

56. Michetti P, Mahan MJ, Slauch JM, Mekalanos JJ, Neutra MR. Monoclonal secretory immunoglobulin A protects mice against oral challenge with the invasive pathogen Salmonella typhimurium. *Infection and immunity* 1992; **60**(5)**:** 1786-1792.

57. Iankov ID, Petrov DP, Mladenov IV, Haralambieva IH, Kalev OK, Balabanova MS *et al.* Protective efficacy of IgA monoclonal antibodies to O and H antigens in a mouse model of intranasal challenge with Salmonella enterica serotype Enteritidis. *Microbes and infection / Institut Pasteur* 2004; **6**(10)**:** 901-910.

58. Alvarez N, Otero O, Camacho F, Borrero R, Tirado Y, Puig A *et al.* Passive administration of purified secretory IgA from human colostrum induces protection against Mycobacterium tuberculosis in a murine model of progressive pulmonary infection. *BMC immunology* 2013; **14 Suppl 1:** S3.

59. Crottet P, Corthésy B. Secretory component delays the conversion of secretory IgA into antigen-binding competent F(ab')2: a possible implication for mucosal defense. *Journal of immunology* 1998; **161**(10)**:** 5445-5453.

60. Duc M, Johansen FE, Corthésy B. Antigen binding to secretory immunoglobulin A results in decreased sensitivity to intestinal proteases and increased binding to cellular Fc receptors. *The Journal of biological chemistry* 2010; **285**(2)**:** 953-960.

61. Phalipon A, Cardona A, Kraehenbuhl JP, Edelman L, Sansonetti PJ, Corthésy B. Secretory component: a new role in secretory IgA-mediated immune exclusion in vivo. *Immunity* 2002; **17**(1)**:** 107-115.

62. Strugnell RA, Wijburg OL. The role of secretory antibodies in infection immunity. *Nature reviews Microbiology* 2010; **8**(9)**:** 656-667.

63. Perrier C, Sprenger N, Corthésy B. Glycans on secretory component participate in innate protection against mucosal pathogens. *The Journal of biological chemistry* 2006; **281**(20)**:** 14280-14287.

64. Robinson JK, Blanchard TG, Levine AD, Emancipator SN, Lamm ME. A mucosal IgA-mediated excretory immune system in vivo. *Journal of immunology* 2001; **166**(6)**:** 3688-3692.

65. Fujioka H, Emancipator SN, Aikawa M, Huang DS, Blatnik F, Karban T *et al.* Immunocytochemical colocalization of specific immunoglobulin A with sendai virus protein in infected polarized epithelium. *The Journal of experimental medicine* 1998; **188**(7)**:** 1223-1229.

66. Fernandez MI, Pedron T, Tournebize R, Olivo-Marin JC, Sansonetti PJ, Phalipon A. Anti-inflammatory role for intracellular dimeric immunoglobulin a by neutralization of lipopolysaccharide in epithelial cells. *Immunity* 2003; **18**(6)**:** 739-749.

67. Boullier S, Tanguy M, Kadaoui KA, Caubet C, Sansonetti P, Corthésy B *et al.* Secretory IgA-mediated neutralization of Shigella flexneri prevents intestinal tissue destruction by down-regulating inflammatory circuits. *Journal of immunology* 2009; **183**(9)**:** 5879-5885.

68. Jennison AV, Verma NK. Shigella flexneri infection: pathogenesis and vaccine development. *FEMS microbiology reviews* 2004; **28**(1)**:** 43-58.

69. Schroeder GN, Hilbi H. Molecular pathogenesis of Shigella spp.: controlling host cell signaling, invasion, and death by type III secretion. *Clinical microbiology reviews* 2008; **21**(1)**:** 134-156.

70. Mounier J, Vasselon T, Hellio R, Lesourd M, Sansonetti PJ. Shigella flexneri enters human colonic Caco-2 epithelial cells through the basolateral pole. *Infection and immunity* 1992; **60**(1)**:** 237-248.

71. Sakaguchi T, Kohler H, Gu X, McCormick BA, Reinecker HC. Shigella flexneri regulates tight junction-associated proteins in human intestinal epithelial cells. *Cellular microbiology* 2002; **4**(6)**:** 367-381.

72. Sasakawa C. A new paradigm of bacteria-gut interplay brought through the study of Shigella. *Proceedings of the Japan Academy Series B, Physical and biological sciences* 2010; **86**(3)**:** 229-243.

73. Kasper CA, Sorg I, Schmutz C, Tschon T, Wischnewski H, Kim ML *et al.* Cell-cell propagation of NF-kappaB transcription factor and MAP kinase activation amplifies innate immunity against bacterial infection. *Immunity* 2010; **33**(5)**:** 804-816.

74. Ashida H, Ogawa M, Mimuro H, Kobayashi T, Sanada T, Sasakawa C. Shigella are versatile mucosal pathogens that circumvent the host innate immune system. *Current opinion in immunology* 2011; **23**(4)**:** 448-455.

75. Sansonetti PJ, Arondel J, Huerre M, Harada A, Matsushima K. Interleukin-8 controls bacterial transepithelial translocation at the cost of epithelial destruction in experimental shigellosis. *Infection and immunity* 1999; **67**(3)**:** 1471-1480.

76. Bahrani FK, Sansonetti PJ, Parsot C. Secretion of Ipa proteins by Shigella flexneri: inducer molecules and kinetics of activation. *Infection and immunity* 1997; **65**(10)**:** 4005-4010.

77. Clerc P, Sansonetti PJ. Entry of Shigella flexneri into HeLa cells: evidence for directed phagocytosis involving actin polymerization and myosin accumulation. *Infection and immunity* 1987; **55**(11)**:** 2681-2688.

78. Watarai M, Tobe T, Yoshikawa M, Sasakawa C. Contact of Shigella with host cells triggers release of Ipa invasins and is an essential function of invasiveness. *The EMBO journal* 1995; **14**(11)**:** 2461-2470.

79. Hanajima-Ozawa M, Matsuzawa T, Fukui A, Kamitani S, Ohnishi H, Abe A *et al.* Enteropathogenic Escherichia coli, Shigella flexneri, and Listeria monocytogenes recruit a junctional protein, zonula occludens-1, to actin tails and pedestals. *Infection and immunity* 2007; **75**(2)**:** 565-573.

80. Voth DE, Ballard JD. Clostridium difficile toxins: mechanism of action and role in disease. *Clinical microbiology reviews* 2005; **18**(2)**:** 247-263.

81. Rasolofo-Razanamparany V, Cassel-Beraud AM, Roux J, Sansonetti PJ, Phalipon A. Predominance of serotype-specific mucosal antibody response in Shigella flexneri-infected humans living in an area of endemicity. *Infection and immunity* 2001; **69**(9)**:** 5230-5234.

82. Clemens JD, Stanton B, Stoll B, Shahid NS, Banu H, Chowdhury AK. Breast feeding as a determinant of severity in shigellosis. Evidence for protection throughout the first three years of life in Bangladeshi children. *American journal of epidemiology* 1986; **123**(4)**:** 710-720.

83. Forbes SJ, Bumpus T, McCarthy EA, Corthésy B, Mantis NJ. Transient suppression of Shigella flexneri type 3 secretion by a protective O-antigen-specific monoclonal IgA. *mBio* 2011; **2**(3)**:** e00042-00011.

84. Keto-Timonen R, Heikinheimo A, Eerola E, Korkeala H. Identification of Clostridium species and DNA fingerprinting of Clostridium perfringens by amplified fragment length polymorphism analysis. *Journal of clinical microbiology* 2006; **44**(11)**:** 4057-4065.

85. Brazier JS, Duerden BI, Hall V, Salmon JE, Hood J, Brett MM *et al.* Isolation and identification of Clostridium spp. from infections associated with the injection of drugs: experiences of a microbiological investigation team. *Journal of medical microbiology* 2002; **51**(11)**:** 985-989.

86. Du T, Alfa MJ. Translocation of Clostridium difficile toxin B across polarized Caco-2 cell monolayers is enhanced by toxin A. *The Canadian journal of infectious diseases = Journal canadien des maladies infectieuses* 2004; **15**(2)**:** 83-88.

87. Sunenshine RH, McDonald LC. Clostridium difficile-associated disease: new challenges from an established pathogen. *Cleveland Clinic journal of medicine* 2006; **73**(2)**:** 187-197.

88. Kachrimanidou M, Malisiovas N. Clostridium difficile infection: a comprehensive review. *Critical reviews in microbiology* 2011; **37**(3)**:** 178-187.

89. Giesemann T, Egerer M, Jank T, Aktories K. Processing of Clostridium difficile toxins. *Journal of medical microbiology* 2008; **57**(Pt 6)**:** 690-696.

90. Teichert M, Tatge H, Schoentaube J, Just I, Gerhard R. Application of mutated Clostridium difficile toxin A for determination of glucosyltransferase-dependent effects. *Infection and immunity* 2006; **74**(10)**:** 6006-6010.

91. Mahida YR, Makh S, Hyde S, Gray T, Borriello SP. Effect of Clostridium difficile toxin A on human intestinal epithelial cells: induction of interleukin 8 production and apoptosis after cell detachment. *Gut* 1996; **38**(3)**:** 337-347.

92. Kim JM, Kim JS, Jun HC, Oh YK, Song IS, Kim CY. Differential expression and polarized secretion of CXC and CC chemokines by human intestinal epithelial cancer cell lines in response to Clostridium difficile toxin A. *Microbiology and immunology* 2002; **46**(5)**:** 333-342.

93. Hecht G, Pothoulakis C, LaMont JT, Madara JL. Clostridium difficile toxin A perturbs cytoskeletal structure and tight junction permeability of cultured human intestinal epithelial monolayers. *The Journal of clinical investigation* 1988; **82**(5)**:** 1516-1524.

94. Nusrat A, von Eichel-Streiber C, Turner JR, Verkade P, Madara JL, Parkos CA. Clostridium difficile toxins disrupt epithelial barrier function by altering membrane microdomain localization of tight junction proteins. *Infection and immunity* 2001; **69**(3)**:** 1329-1336.

95. Johnson S, Gerding DN, Janoff EN. Systemic and mucosal antibody responses to toxin A in patients infected with Clostridium difficile. *The Journal of infectious diseases* 1992; **166**(6)**:** 1287-1294.

96. Kyne L, Warny M, Qamar A, Kelly CP. Association between antibody response to toxin A and protection against recurrent Clostridium difficile diarrhoea. *Lancet* 2001; **357**(9251)**:** 189-193.

97. Abougergi MS, Kwon JH. Intravenous immunoglobulin for the treatment of Clostridium difficile infection: a review. *Digestive diseases and sciences* 2011; **56**(1)**:** 19-26.

98. Kink JA, Williams JA. Antibodies to recombinant Clostridium difficile toxins A and B are an effective treatment and prevent relapse of C. difficile-associated disease in a hamster model of infection. *Infection and immunity* 1998; **66**(5)**:** 2018-2025.

99. Johnson S, Sypura WD, Gerding DN, Ewing SL, Janoff EN. Selective neutralization of a bacterial enterotoxin by serum immunoglobulin A in response to mucosal disease. *Infection and immunity* 1995; **63**(8)**:** 3166-3173.

100. Lowy I, Molrine DC, Leav BA, Blair BM, Baxter R, Gerding DN *et al.* Treatment with monoclonal antibodies against Clostridium difficile toxins. *The New England journal of medicine* 2010; **362**(3)**:** 197-205.

101. Wilton JM. Suppression by IgA of IgG-mediated phagocytosis by human polymorphonuclear leucocytes. *Clinical and experimental immunology* 1978; **34**(3)**:** 423-428.

102. Nikolova EB, Russell MW. Dual function of human IgA antibodies: inhibition of phagocytosis in circulating neutrophils and enhancement of responses in IL-8-stimulated cells. *Journal of leukocyte biology* 1995; **57**(6)**:** 875-882.

103. Wolf HM, Fischer MB, Puhringer H, Samstag A, Vogel E, Eibl MM. Human serum IgA downregulates the release of inflammatory cytokines (tumor necrosis factor-alpha, interleukin-6) in human monocytes. *Blood* 1994; **83**(5)**:** 1278-1288.

104. Carayannopoulos L, Hexham JM, Capra JD. Localization of the binding site for the monocyte immunoglobulin (Ig) A-Fc receptor (CD89) to the domain boundary between Calpha2 and Calpha3 in human IgA1. *The Journal of experimental medicine* 1996; **183**(4)**:** 1579-1586.

105. Pleass RJ, Dunlop JI, Anderson CM, Woof JM. Identification of residues in the CH2/CH3 domain interface of IgA essential for interaction with the human fcalpha receptor (FcalphaR) CD89. *The Journal of biological chemistry* 1999; **274**(33)**:** 23508-23514.

106. van der Boog PJ, van Zandbergen G, de Fijter JW, Klar-Mohamad N, van Seggelen A, Brandtzaeg P *et al.* Fc alpha RI/CD89 circulates in human serum covalently linked to IgA in a polymeric state. *Journal of immunology* 2002; **168**(3)**:** 1252-1258.

107. Launay P, Grossetete B, Arcos-Fajardo M, Gaudin E, Torres SP, Beaudoin L *et al.* Fcalpha receptor (CD89) mediates the development of immunoglobulin A (IgA) nephropathy (Berger's disease). Evidence for pathogenic soluble receptor-Iga complexes in patients and CD89 transgenic mice. *The Journal of experimental medicine* 2000; **191**(11)**:** 1999-2009.

108. Kaetzel CS. The polymeric immunoglobulin receptor: bridging innate and adaptive immune responses at mucosal surfaces. *Immunological reviews* 2005; **206:** 83-99.

109. Asano M, Komiyama K. Polymeric immunoglobulin receptor. *Journal of oral science* 2011; **53**(2)**:** 147-156.

110. Johansen FE, Kaetzel CS. Regulation of the polymeric immunoglobulin receptor and IgA transport: new advances in environmental factors that stimulate pIgR expression and its role in mucosal immunity. *Mucosal immunology* 2011; **4**(6)**:** 598-602.

111. Shibuya A, Sakamoto N, Shimizu Y, Shibuya K, Osawa M, Hiroyama T *et al.* Fc alpha/mu receptor mediates endocytosis of IgM-coated microbes. *Nature immunology* 2000; **1**(5)**:** 441-446.

112. Wang R, Fu Y, Zhao Q, Pan L, Zhang W. Human Fcalpha/muR and pIgR distribute differently in intestinal tissues. *Biochemical and biophysical research communications* 2009; **381**(2)**:** 148-152.

113. Ghumra A, Shi J, McIntosh RS, Rasmussen IB, Braathen R, Johansen FE *et al.* Structural requirements for the interaction of human IgM and IgA with the human Fcalpha/mu receptor. *European journal of immunology* 2009; **39**(4)**:** 1147-1156.

114. Marsee DK, Pinkus GS, Yu H. CD71 (transferrin receptor): an effective marker for erythroid precursors in bone marrow biopsy specimens. *American journal of clinical pathology* 2010; **134**(3)**:** 429-435.

115. Moura IC, Centelles MN, Arcos-Fajardo M, Malheiros DM, Collawn JF, Cooper MD *et al.* Identification of the transferrin receptor as a novel immunoglobulin (Ig)A1 receptor and its enhanced expression on mesangial cells in IgA nephropathy. *The Journal of experimental medicine* 2001; **194**(4)**:** 417-425.

116. Matysiak-Budnik T, Moura IC, Arcos-Fajardo M, Lebreton C, Menard S, Candalh C *et al.* Secretory IgA mediates retrotranscytosis of intact gliadin peptides via the transferrin receptor in celiac disease. *The Journal of experimental medicine* 2008; **205**(1)**:** 143-154.

117. Lebreton C, Menard S, Abed J, Moura IC, Coppo R, Dugave C *et al.* Interactions among secretory immunoglobulin A, CD71, and transglutaminase-2 affect permeability of intestinal epithelial cells to gliadin peptides. *Gastroenterology* 2012; **143**(3)**:** 698-707 e691-694.

118. Abed J, Lebreton C, Champier G, Cuvillier A, Cogne M, Meresse B *et al.* Abnormal apical-to-basal transport of dietary ovalbumin by secretory IgA stimulates a mucosal Th1 response. *Mucosal immunology* 2013.

119. Yang W, Mou T, Shao G, Wang F, Zhang X, Liu B. Copolymer-based hepatocyte asialoglycoprotein receptor targeting agent for SPECT. *Journal of nuclear medicine : official publication, Society of Nuclear Medicine* 2011; **52**(6)**:** 978-985.

120. Monteiro RC, Van De Winkel JG. IgA Fc receptors. *Annual review of immunology* 2003; **21:** 177-204.

121. Mantis NJ, Cheung MC, Chintalacharuvu KR, Rey J, Corthésy B, Neutra MR. Selective adherence of IgA to murine Peyer's patch M cells: evidence for a novel IgA receptor. *Journal of immunology* 2002; **169**(4)**:** 1844-1851.

122. Rochereau N, Drocourt D, Perouzel E, Pavot V, Redelinghuys P, Brown GD *et al.* Dectin-1 is essential for reverse transcytosis of glycosylated SIgA-antigen complexes by intestinal M cells. *PLoS biology* 2013; **11**(9)**:** e1001658.

123. Lamkhioued B, Gounni AS, Gruart V, Pierce A, Capron A, Capron M. Human eosinophils express a receptor for secretory component. Role in secretory IgA-dependent activation. *European journal of immunology* 1995; **25**(1)**:** 117-125.

124. Iikura M, Yamaguchi M, Fujisawa T, Miyamasu M, Takaishi T, Morita Y *et al.* Secretory IgA induces degranulation of IL-3-primed basophils. *Journal of immunology* 1998; **161**(3)**:** 1510-1515.

125. Ehrhardt GR, Hsu JT, Gartland L, Leu CM, Zhang S, Davis RS *et al.* Expression of the immunoregulatory molecule FcRH4 defines a distinctive tissue-based population of memory B cells. *The Journal of experimental medicine* 2005; **202**(6)**:** 783-791.

126. Falini B, Tiacci E, Pucciarini A, Bigerna B, Kurth J, Hatzivassiliou G *et al.* Expression of the IRTA1 receptor identifies intraepithelial and subepithelial marginal zone B cells of the mucosa-associated lymphoid tissue (MALT). *Blood* 2003; **102**(10)**:** 3684-3692.

127. Ehrhardt GR, Davis RS, Hsu JT, Leu CM, Ehrhardt A, Cooper MD. The inhibitory potential of Fc receptor homolog 4 on memory B cells. *Proceedings of the National Academy of Sciences of the United States of America* 2003; **100**(23)**:** 13489-13494.

128. Moir S, Ho J, Malaspina A, Wang W, DiPoto AC, O'Shea MA *et al.* Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals. *The Journal of experimental medicine* 2008; **205**(8)**:** 1797-1805.

129. Klimovich VB. IgM and its receptors: structural and functional aspects. *Biochemistry Biokhimiia* 2011; **76**(5)**:** 534-549.

130. Wilson TJ, Fuchs A, Colonna M. Cutting edge: human FcRL4 and FcRL5 are receptors for IgA and IgG. *Journal of immunology* 2012; **188**(10)**:** 4741-4745.

131. Mota G, Manciulea M, Cosma E, Popescu I, Hirt M, Jensen-Jarolim E *et al.* Human NK cells express Fc receptors for IgA which mediate signal transduction and target cell killing. *European journal of immunology* 2003; **33**(8)**:** 2197-2205.

132. Phillips-Quagliata JM, Patel S, Han JK, Arakelov S, Rao TD, Shulman MJ *et al.* The IgA/IgM receptor expressed on a murine B cell lymphoma is poly-Ig receptor. *Journal of immunology* 2000; **165**(5)**:** 2544-2555.

133. Croft NM, Hodges M. IgM: mucosal response in acute diarrhoeal disease of infants. *Scandinavian journal of gastroenterology* 2005; **40**(8)**:** 965-971.

134. Ehrenstein MR, Notley CA. The importance of natural IgM: scavenger, protector and regulator. *Nature reviews Immunology* 2010; **10**(11)**:** 778-786.

135. Arnold JN, Wormald MR, Suter DM, Radcliffe CM, Harvey DJ, Dwek RA *et al.* Human serum IgM glycosylation: identification of glycoforms that can bind to mannan-binding lectin. *The Journal of biological chemistry* 2005; **280**(32)**:** 29080-29087.

136. Czajkowsky DM, Shao Z. The human IgM pentamer is a mushroom-shaped molecule with a flexural bias. *Proceedings of the National Academy of Sciences of the United States of America* 2009; **106**(35)**:** 14960-14965.

137. Muller R, Grawert MA, Kern T, Madl T, Peschek J, Sattler M *et al.* High-resolution structures of the IgM Fc domains reveal principles of its hexamer formation. *Proceedings of the National Academy of Sciences of the United States of America* 2013; **110**(25)**:** 10183-10188.

138. Feinstein A, Munn EA. Conformation of the free and antigen-bound IgM antibody molecules. *Nature* 1969; **224**(5226)**:** 1307-1309.

139. Perkins SJ, Nealis AS, Sutton BJ, Feinstein A. Solution structure of human and mouse immunoglobulin M by synchrotron X-ray scattering and molecular graphics modelling. A possible mechanism for complement activation. *Journal of molecular biology* 1991; **221**(4)**:** 1345-1366.

140. Brandtzaeg P. Human secretory immunoglobulin M. An immunochemical and immunohistochemical study. *Immunology* 1975; **29**(3)**:** 559-570.

141. Longet S, Miled S, Lotscher M, Miescher SM, Zuercher AW, Corthésy B. Human plasma-derived polymeric IgA and IgM antibodies associate with secretory component to yield biologically active secretory-like antibodies. *The Journal of biological chemistry* 2013; **288**(6)**:** 4085-4094.

142. Norderhaug IN, Johansen FE, Krajci P, Brandtzaeg P. Domain deletions in the human polymeric Ig receptor disclose differences between its dimeric IgA and pentameric IgM interaction. *European journal of immunology* 1999; **29**(10)**:** 3401-3409.

143. Prinsloo E, Oosthuizen V, Van de Venter M, Naude RJ. Biological inferences from IgM binding characteristics of recombinant human secretory component mutants. *Immunology letters* 2009; **122**(1)**:** 94-98.

144. Boes M, Esau C, Fischer MB, Schmidt T, Carroll M, Chen J. Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. *Journal of immunology* 1998; **160**(10)**:** 4776-4787.

145. Ehrenstein MR, O'Keefe TL, Davies SL, Neuberger MS. Targeted gene disruption reveals a role for natural secretory IgM in the maturation of the primary immune response. *Proceedings of the National Academy of Sciences of the United States of America* 1998; **95**(17)**:** 10089-10093.

146. Boes M, Prodeus AP, Schmidt T, Carroll MC, Chen J. A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection. *The Journal of experimental medicine* 1998; **188**(12)**:** 2381-2386.

147. Salinas-Carmona MC, Perez-Rivera I. Humoral immunity through immunoglobulin M protects mice from an experimental actinomycetoma infection by Nocardia brasiliensis. *Infection and immunity* 2004; **72**(10)**:** 5597-5604.

148. Alugupalli KR, Gerstein RM, Chen J, Szomolanyi-Tsuda E, Woodland RT, Leong JM. The resolution of relapsing fever borreliosis requires IgM and is concurrent with expansion of B1b lymphocytes. *Journal of immunology* 2003; **170**(7)**:** 3819-3827.

149. Brown JS, Hussell T, Gilliland SM, Holden DW, Paton JC, Ehrenstein MR *et al.* The classical pathway is the dominant complement pathway required for innate immunity to Streptococcus pneumoniae infection in mice. *Proceedings of the National Academy of Sciences of the United States of America* 2002; **99**(26)**:** 16969-16974.

150. Kinoshita M, Shinomiya N, Ono S, Tsujimoto H, Kawabata T, Matsumoto A *et al.* Restoration of natural IgM production from liver B cells by exogenous IL-18 improves the survival of burn-injured mice infected with Pseudomonas aeruginosa. *Journal of immunology* 2006; **177**(7)**:** 4627-4635.

151. Baumgarth N, Herman OC, Jager GC, Brown LE, Herzenberg LA, Chen J. B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. *The Journal of experimental medicine* 2000; **192**(2)**:** 271-280.

152. Jayasekera JP, Moseman EA, Carroll MC. Natural antibody and complement mediate neutralization of influenza virus in the absence of prior immunity. *Journal of virology* 2007; **81**(7)**:** 3487-3494.

153. Choi YS, Baumgarth N. Dual role for B-1a cells in immunity to influenza virus infection. *The Journal of experimental medicine* 2008; **205**(13)**:** 3053-3064.

154. Diamond MS, Sitati EM, Friend LD, Higgs S, Shrestha B, Engle M. A critical role for induced IgM in the protection against West Nile virus infection. *The Journal of experimental medicine* 2003; **198**(12)**:** 1853-1862.

155. Subramaniam KS, Datta K, Quintero E, Manix C, Marks MS, Pirofski LA. The absence of serum IgM enhances the susceptibility of mice to pulmonary challenge with Cryptococcus neoformans. *Journal of immunology* 2010; **184**(10)**:** 5755-5767.

156. Couper KN, Phillips RS, Brombacher F, Alexander J. Parasite-specific IgM plays a significant role in the protective immune response to asexual erythrocytic stage Plasmodium chabaudi AS infection. *Parasite immunology* 2005; **27**(5)**:** 171-180.

157. Couper KN, Roberts CW, Brombacher F, Alexander J, Johnson LL. Toxoplasma gondii-specific immunoglobulin M limits parasite dissemination by preventing host cell invasion. *Infection and immunity* 2005; **73**(12)**:** 8060-8068.

158. Baral TN, De Baetselier P, Brombacher F, Magez S. Control of Trypanosoma evansi infection is IgM mediated and does not require a type I inflammatory response. *The Journal of infectious diseases* 2007; **195**(10)**:** 1513-1520.

159. Fabrizio K, Groner A, Boes M, Pirofski LA. A human monoclonal immunoglobulin M reduces bacteremia and inflammation in a mouse model of systemic pneumococcal infection. *Clinical and vaccine immunology : CVI* 2007; **14**(4)**:** 382-390.

160. Secher T, Fauconnier L, Szade A, Rutschi O, Fas SC, Ryffel B *et al.* Anti-Pseudomonas aeruginosa serotype O11 LPS immunoglobulin M monoclonal antibody panobacumab (KBPA101) confers protection in a murine model of acute lung infection. *The Journal of antimicrobial chemotherapy* 2011; **66**(5)**:** 1100-1109.

161. Nouir NB, Eschbach ML, Piedavent M, Osterloh A, Kingsley MT, Erttmann K *et al.* Vaccination with Strongyloides ratti heat shock protein 60 increases susceptibility to challenge infection by induction of Th1 response. *Vaccine* 2012; **30**(5)**:** 862-871.

162. Lachmann RA, van Kaam AH, Haitsma JJ, Verbrugge SJ, Delreu F, Lachmann B. Immunoglobulin M-enriched intravenous polyclonal immunoglobulins reduce bacteremia following Klebsiella pneumoniae infection in an acute respiratory distress syndrome rat model. *Experimental lung research* 2004; **30**(4)**:** 251-260.

163. Stehr SN, Knels L, Weissflog C, Schober J, Haufe D, Lupp A *et al.* Effects of IGM-enriched solution on polymorphonuclear neutrophil function, bacterial clearance, and lung histology in endotoxemia. *Shock* 2008; **29**(2)**:** 167-172.

164. Norrby-Teglund A, Haque KN, Hammarstrom L. Intravenous polyclonal IgM-enriched immunoglobulin therapy in sepsis: a review of clinical efficacy in relation to microbiological aetiology and severity of sepsis. *Journal of internal medicine* 2006; **260**(6)**:** 509-516.

165. Toth I, Mikor A, Leiner T, Molnar Z, Bogar L, Szakmany T. Effects of IgM-enriched immunoglobulin therapy in septic-shock-induced multiple organ failure: pilot study. *Journal of anesthesia* 2013; **27**(4)**:** 618-622.

166. Brunner R, Rinner W, Haberler C, Kitzberger R, Sycha T, Herkner H *et al.* Early treatment with IgM-enriched intravenous immunoglobulin does not mitigate critical illness polyneuropathy and/or myopathy in patients with multiple organ failure and SIRS/sepsis: a prospective, randomized, placebo-controlled, double-blinded trial. *Critical care* 2013; **17**(5)**:** R213.

167. Haque KN, Zaidi MH, Bahakim H. IgM-enriched intravenous immunoglobulin therapy in neonatal sepsis. *American journal of diseases of children* 1988; **142**(12)**:** 1293-1296.

168. Capasso L, Borrelli AC, Parrella C, Lama S, Ferrara T, Coppola C *et al.* Are IgM-enriched immunoglobulins an effective adjuvant in septic VLBW infants? *Italian journal of pediatrics* 2013; **39:** 63.

169. Schedel I, Dreikhausen U, Nentwig B, Hockenschnieder M, Rauthmann D, Balikcioglu S *et al.* Treatment of gram-negative septic shock with an immunoglobulin preparation: a prospective, randomized clinical trial. *Critical care medicine* 1991; **19**(9)**:** 1104-1113.

170. Behre G, Schedel I, Nentwig B, Wormann B, Essink M, Hiddemann W. Endotoxin concentration in neutropenic patients with suspected gram-negative sepsis: correlation with clinical outcome and determination of anti-endotoxin core antibodies during therapy with polyclonal immunoglobulin M-enriched immunoglobulins. *Antimicrobial agents and chemotherapy* 1992; **36**(10)**:** 2139-2146.

171. Jackson SK, Parton J, Barnes RA, Poynton CH, Fegan C. Effect of IgM-enriched intravenous immunoglobulin (Pentaglobin) on endotoxaemia and anti-endotoxin antibodies in bone marrow transplantation. *European journal of clinical investigation* 1993; **23**(9)**:** 540-545.

172. Norrby-Teglund A, Ihendyane N, Kansal R, Basma H, Kotb M, Andersson J *et al.* Relative neutralizing activity in polyspecific IgM, IgA, and IgG preparations against group A streptococcal superantigens. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2000; **31**(5)**:** 1175-1182.

173. Raedler A, Ladehoff E, Schug S, Schreiber S, Greten H. Adjuvante therapie des Morbus Crohn mit IgM angereichertem Immunoglobulin. *Klinisch angewandte Immunologie: Sepsistherapie* 1986**:** 50-58.

174. Rogosnitzky M, Danks R, Holt D. Intravenous immunoglobulin for the treatment of Crohn's disease. *Autoimmunity reviews* 2012; **12**(2)**:** 275-280.

175. Raedler A, Ladehof E, Schug S, Schreiber S, Greten J. Therapy of Crohn's disease with IgM-enriched immunoglobulin. *Gastroenterology* 1988; **94:** A363.

176. Rieben R, Roos A, Muizert Y, Tinguely C, Gerritsen AF, Daha MR. Immunoglobulin M-enriched human intravenous immunoglobulin prevents complement activation in vitro and in vivo in a rat model of acute inflammation. *Blood* 1999; **93**(3)**:** 942-951.

177. Yoo EM, Trinh KR, Lim H, Wims LA, Morrison SL. Characterization of IgA and IgM binding and internalization by surface-expressed human Fcalpha/mu receptor. *Molecular immunology* 2011; **48**(15-16)**:** 1818-1826.

178. Kubagawa H, Oka S, Kubagawa Y, Torii I, Takayama E, Kang DW *et al.* Identity of the elusive IgM Fc receptor (FcmuR) in humans. *The Journal of experimental medicine* 2009; **206**(12)**:** 2779-2793.

179. Chapel H, Gardulf A. Subcutaneous immunoglobulin replacement therapy: the European experience. *Current opinion in allergy and clinical immunology* 2013; **13**(6)**:** 623-629.

180. Ballow M. Primary immunodeficiency disorders: antibody deficiency. *The Journal of allergy and clinical immunology* 2002; **109**(4)**:** 581-591.

181. Schwab I, Nimmerjahn F. Intravenous immunoglobulin therapy: how does IgG modulate the immune system? *Nature reviews Immunology* 2013; **13**(3)**:** 176-189.

182. Orange JS, Hossny EM, Weiler CR, Ballow M, Berger M, Bonilla FA *et al.* Use of intravenous immunoglobulin in human disease: a review of evidence by members of the Primary Immunodeficiency Committee of the American Academy of Allergy, Asthma and Immunology. *The Journal of allergy and clinical immunology* 2006; **117**(4 Suppl)**:** S525-553.

183. McPherson S, Rees CJ, Ellis R, Soo S, Panter SJ. Intravenous immunoglobulin for the treatment of severe, refractory, and recurrent Clostridium difficile diarrhea. *Diseases of the colon and rectum* 2006; **49**(5)**:** 640-645.

184. Cramer M, Frei R, Sebald A, Mazzoletti P, Maeder W. Stability over 36 months of a new liquid 10% polyclonal immunoglobulin product (IgPro10, Privigen) stabilized with L-proline. *Vox sanguinis* 2009; **96**(3)**:** 219-225.

185. Ferrara G, Zumla A, Maeurer M. Intravenous immunoglobulin (IVIg) for refractory and difficult-to-treat infections. *The American journal of medicine* 2012; **125**(10)**:** 1036 e1031-1038.

186. Gardulf A, Hammarstrom L, Smith CI. Home treatment of hypogammaglobulinaemia with subcutaneous gammaglobulin by rapid infusion. *Lancet* 1991; **338**(8760)**:** 162-166.

187. Gardulf A, Andersen V, Bjorkander J, Ericson D, Froland SS, Gustafson R *et al.* Subcutaneous immunoglobulin replacement in patients with primary antibody deficiencies: safety and costs. *Lancet* 1995; **345**(8946)**:** 365-369.

188. Fasth A, Nystrom J. Safety and efficacy of subcutaneous human immunoglobulin in children with primary immunodeficiency. *Acta paediatrica* 2007; **96**(10)**:** 1474-1478.

189. Gardulf A, Bjorvell H, Andersen V, Bjorkander J, Ericson D, Froland SS *et al.* Lifelong treatment with gammaglobulin for primary antibody deficiencies: the patients' experiences of subcutaneous self-infusions and home therapy. *Journal of advanced nursing* 1995; **21**(5)**:** 917-927.

190. Duhem C, Dicato MA, Ries F. Side-effects of intravenous immune globulins. *Clinical and experimental immunology* 1994; **97 Suppl 1:** 79-83.

191. Gregersen S, Aalokken TM, Mynarek G, Kongerud J, Aukrust P, Froland SS *et al.* High resolution computed tomography and pulmonary function in common variable immunodeficiency. *Respiratory medicine* 2009; **103**(6)**:** 873-880.

192. Kalha I, Sellin JH. Common variable immunodeficiency and the gastrointestinal tract. *Current gastroenterology reports* 2004; **6**(5)**:** 377-383.

193. Honda K, Littman DR. The microbiome in infectious disease and inflammation. *Annual review of immunology* 2012; **30:** 759-795.

194. McFarland LV. What's lurking under the bed? Persistence and predominance of particular Clostridium difficile strains in a hospital and the potential role of environmental contamination. *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America* 2002; **23**(11)**:** 639-640.

195. Favre LI, Spertini F, Corthésy B. Simplified procedure to recover recombinant antigenized secretory IgA to be used as a vaccine vector. *Journal of chromatography B, Analytical technologies in the biomedical and life sciences* 2003; **786**(1-2)**:** 143-151.

196. Thierry AC, Bernasconi E, Mercenier A, Corthésy B. Conditioned polarized Caco-2 cell monolayers allow to discriminate for the ability of gut-derived microorganisms to modulate permeability and antigen-induced basophil degranulation. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 2009; **39**(4)**:** 527-536.

197. Cottet S, Corthésy-Theulaz I, Spertini F, Corthésy B. Microaerophilic conditions permit to mimic in vitro events occurring during in vivo Helicobacter pylori infection and to identify Rho/Ras-associated proteins in cellular signaling. *The Journal of biological chemistry* 2002; **277**(37)**:** 33978-33986.

198. Kadaoui KA, Corthésy B. Secretory IgA mediates bacterial translocation to dendritic cells in mouse Peyer's patches with restriction to mucosal compartment. *Journal of immunology* 2007; **179**(11)**:** 7751-7757.

199. Bollinger RR, Everett ML, Palestrant D, Love SD, Lin SS, Parker W. Human secretory immunoglobulin A may contribute to biofilm formation in the gut. *Immunology* 2003; **109**(4)**:** 580-587.