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Development of an opsonophagocytic assay for the measurement of functional antibody activity against *Bordetella pertussis*

Vývoj opsonofagocytárního testu pro měření funkční aktivity protilátek proti *Bordetella pertussis*

Diploma thesis / Diplomová práce

Školitel:

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Abstract

The Gram-negative pathogen bacterium *Bordetella pertussis* is the infectious agent causing pertussis or whooping cough. The infection is dangerous to infants, often being deadly if untreated. Since whole-cell pertussis vaccines have been replaced by acellular pertussis vaccines, pertussis has become the most prevalent vaccine-preventable disease in developed countries. Therefore, the development of a new generation of pertussis vaccines has become a high priority. Opsonophagocytic assays are one method used to assess the efficacy of new vaccines. The main objective of the thesis is to develop opsonophagocytic killing and uptake assays for the measurement of functional antibody activity against *Bordetella pertussis*. Neutrophils from mice and humans were isolated by three different methods and used for the assessment of different human and mouse sera in opsonophagocytic killing and uptake assays. Different experimental conditions were tested, including multiplicity of infection and serum dilutions. The opsonophagocytic uptake assay proved to discriminate between naïve and immune sera. Serum from mice vaccinated with the whole-cell pertussis vaccine enhanced opsonophagocytic uptake of *B. pertussis* cells into neutrophils, while serum from mice immunized with the acellular pertussis vaccine did not. These data are in agreement with observations showing a lower efficacy of acellular pertussis vaccines.

Key words: *Bordetella pertussis*, vaccine, opsonophagocytosis, antibody, neutrophil

Abstrakt

Gram-negativní bakterie *Bordetella pertussis* je pathogen způsobující onemocnění zvané pertuse nebo černý kašel. Infekce je zvláště nebezpečná pro kojence a pokud není léčena, může být pro ně až smrtelná. Po zavedení acelulární pertusové vakcíny místo celobuněčné dochází k nárůstu případů onemocnění a pertuse je v rozvinutých zemích nejrozšířenější nemoc, jíž lze předejít očkováním. Z toho důvodu je důležitý vývoj nové generace vakcín a stoupá i důležitost metod vyhodnocujících účinnost vakcín. Měření opsonofagocytických procesů je jedna z metod využívaná k vyhodnocení odpovědi na vakcinaci. Hlavní cíl této práce je vývoj opsonofagocytických testů, které měří pohlcení částic a zabíjení mikroorganizmů pro měření funkčních vlastností protilátek proti *Bordetella pertussis*. Myší a lidské neutrofily byly izolovány třemi různými metodami, jejich opsonofgocytická aktivita byla porovnána a byly použity pro zhodnocení lidských a myších sér v opsonofagocytických testech. Různé experimentální podmínky opsonofagocytických testů byly testovány, včetně různých poměrů fagocytických buněk k bakteriálním buňkám a různých ředění sér. Opsonofagocytický test rozlišil naivní od imunizovaného séra. Sérum z myší vakcinovaných celobuněčnou vakcínou zvýšilo účinnost fagocytózy, kdežto to z myší vakcinovaných acelulární vakcínou účinnost fagocytózy nezvyšovalo. Tato data jsou v souladu s obecně pozorovanou nízkou účinností acelulárních vakcín.

Klíčová slova: *Bordetella pertussis*, vakcína, opsonofagocytóza, protilátka, neutrofil

Abbreviations

ACT	adenylate cyclase toxin
aP	acellular pertussis
BP	*Bordetella pertussis*
Bvg	*Bordetella* virulence-associated genes
CFU	colony forming unit
DNT	dermonecrotic toxin
ELISA	enzyme-linked immunosorbent assay
FHA	filamentous hemagglutinin
FIM	fimbriae
HI	heat-inactivated
HRP	horseradish peroxidase
mAb	monoclonal antibody
MFI	mean fluorescent intensity
MOI	multiplicity of infection
NET	neutrophil extracellular trap
OPA	opsonophagocytic uptake assay
OPK	opsonophagocytic killing assay
PBMN	peripheral blood mononuclear cells
PRN	pertactin
PT	pertussis toxin
TCT	tracheal cytotoxin
wP	whole-cell pertussis
WT	wild type

Contents

1. Introduction and literature review

Infection by *Bordetella pertussis*, the gram-negative human pathogen causing whooping cough, has been thought to be fully preventable by vaccination. Recent data suggest that incidence of whooping cough cases is increasing, with causes for this increase being found in the changes of vaccine composition and also pathogen evolution. *B. pertussis* expresses many virulence factors, many of which are not included in current vaccines. The evaluation of vaccine efficacy is becoming more important to limit infection spreading and manifestation of symptoms.

Assessment of phagocytosis is a method used for analysis of response to vaccination. Opsonophagocytic assays, so termed because they combine the effect of serum, antibody or complement opsonization with phagocytosis, are used for such measurements. Two general types of opsonophagocytic assays are recognized: first, assays evaluating uptake of target particles and second, assays quantifying the killing of target microorganisms.

1.1.   *Bordetella pertussis* and its virulence factors

The obligatory human pathogen this thesis focuses on is *Bordetella pertussis*, a gram-negative bacterium. *B. pertussis* is recognized as the causative agent of whooping cough or pertussis, a disease of the lower respiratory tract. This disease is characterized by three stages. An early catarrhal phase presents with symptoms similar to the common cold, ranging from rhinorrhea to mild cough. The second stage manifests the typical paroxysmal cough, which gave the disease its name. The bouts of coughing limit breathing and may result in vomiting. The last stage, the convalescent phase, is a recovery of the immune system (Zee et al., 2015). Before vaccination usage, whooping cough was a significant cause of infant mortality. The disease had been considered fully preventable by vaccination. A very efficient vaccination program involving immunization with a whole-cell vaccine (wP) led to dramatic reduction of disease incidence. In the 1980s and 1990s, the whole-cell vaccine was replaced with acellular vaccine in many countries. Following this, a resurgence of pertussis incidence has been marked, with many epidemics since 2000 (Tan et al., 2015; Warfel et al., 2014).

The pathogenesis caused by *Bordetella pertussis* is complex, being mediated by a variety of virulence factors. Among the notoriously known are toxins. Pertussis toxin (PT) is an exotoxin acting through heterotrimeric G proteins, causing dysregulation of early neutrophil response (Carbonetti et al., 2003; Kirimanjeswara et al., 2005; Weiss et al., 1984). Another important exotoxin is the adenylate cyclase toxin (ACT), which binds to complement receptor 3 (CD11b/CD18, $\alpha_M\beta_2$ or Mac-1) and catalyzes massive cAMP production, thus leading to target

cell signaling deregulation (Osicka et al., 2015; Sebo et al., 2014; Weiss et al., 1984). Other toxins produced by *Bordetella pertussis* are the dermonecrotic toxin (DNT), and tracheal cytotoxin (TCT). Adhesins are also important virulence factors, mediating attachment of the bacteria to host cell and some immune system evasion strategies. Among these belong filamentous hemagglutinin (FHA), pertactin (PRN) and fimbriae (FIM) of type 2 (FIM2) and 3 (FIM3) (Rodriguez et al., 2006). Among other virulence factors are BrkA and Vag8, important for resistance against serum complement-dependent killing (Brookes et al., 2018; Fernandez and Weiss, 1994). The *Bordetella* virulence factor expression is under the regulation of a two-component system BvgA/S. During the Bvg$^+$ phase, many virulence activated genes (*vags*) are expressed, in the Bvg$^-$ phase, which is supposedly important for the spreading of infection, the *vags* are repressed. Instead, virulence repressed genes (*vrgs*) are active. Less understood is an intermediate phase, known as Bvg^i (Coutte et al., 2016; Stockbauer et al., 2001).
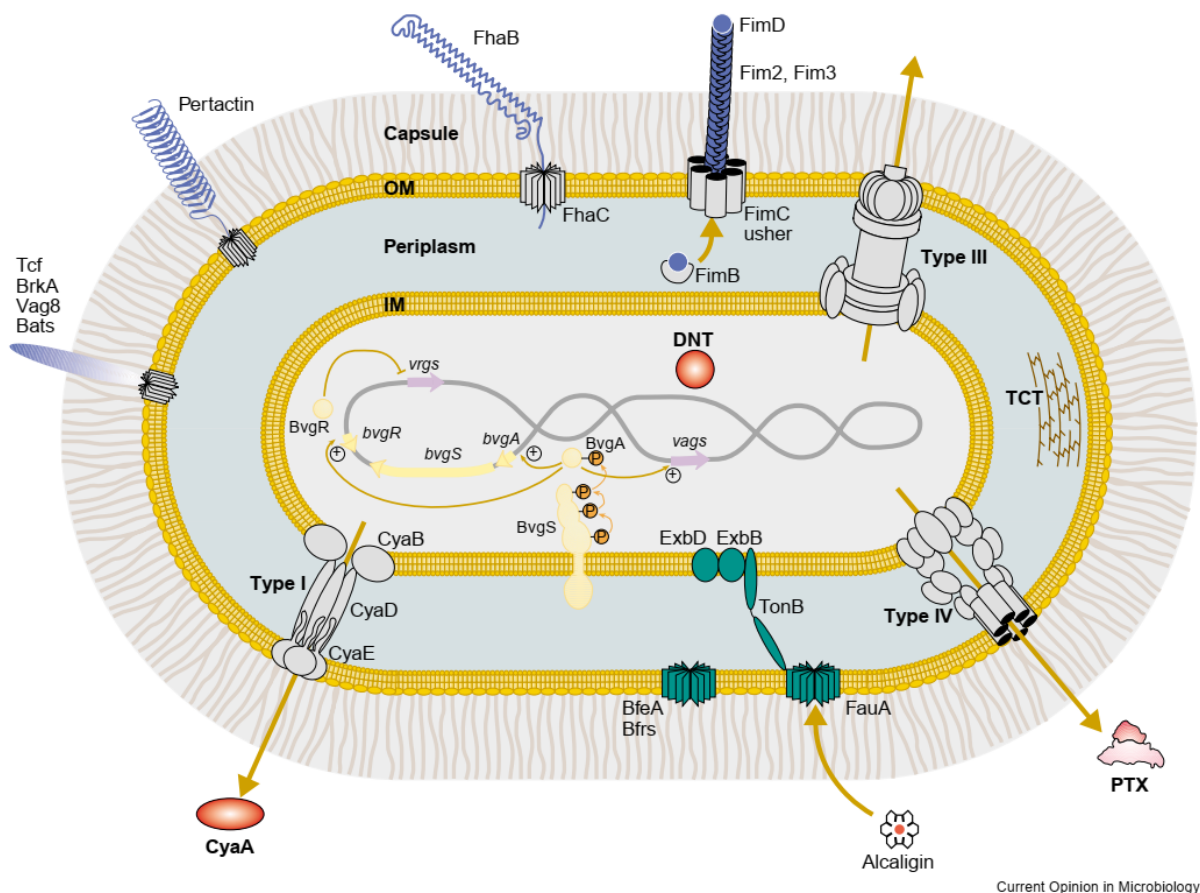
Figure 1 – Virulence factors of *Bordetella pertussis* – Schematic depiction of cellular structures (OM – outer membrane, IM – inner membrane) and virulence factors. Adhesins are depicted in blue color (Fim – Fimbriae subunits, FhaB – filamentous hemagglitinin subunit). Toxins are depicted in red color (CyaA – adenylate cyclase toxin, PTX – pertussis toxin, TCT – tracheal cytotoxin, DNT – dermonecrotic toxin). Transport proteins are depicted in gray color. Taken from (Locht et al., 2001)

1.2. Vaccination against *B. pertussis*

Whole-cell vaccine consists of whole, inactivated *Bordetella pertussis* cells with adjuvant, most often alum. Acellular vaccines are comprised of purified components of the bacterium and adjuvant. Among the most often included antigens are pertussis toxin, pertactin, filamentous hemagglutinin and fimbriae (Antunes et al., 2018). The number of antigens included and their concentration varies greatly between individual manufacturers (Queenan et al., 2019; Raeven et al., 2019). The vaccine is often combined with diphtheria and tetanus toxoid (Antunes et al., 2018). For example, two vaccines available in the Czech Republic for vaccination of infants are Hexacima and Infanrix Hexa. Hexacima includes diphtheria toxoid, tetanus toxoid, inactivated poliovirus, hepatitis B surface antigen, *Haemophilus influenzae* polysaccharide and *B. pertussis* antigens pertussis toxoid and filamentous hemagglutinin. Infanrix Hexa includes the same antigens as Hexacima, plus *B. pertussis* pertactin ("Hexacima | European Medicines Agency," 2019; "Infanrix Hexa | European Medicines Agency," 2019).

Table 1 – Component overview of acellular pertussis vaccines – Content of immunogens in µg or IU. D – diptheria, T – tetanus, aP – acellular pertussis, PT – pertussis toxin, FHA – filamentous hemagglutinin, PRN – pertactin, FIM2/3 – fimbriae type 2/3, DT – diptheria toxin, TT – tetanus toxin, IU – international units, Lf – liquid flocculation units. Infanrix Hexa®, Pentacel® and Hexacima also contain other components than those listed. Adapted from Queenan et al., 2019, "Hexacima | European Medicines Agency," 2019; "Infanrix Hexa | European Medicines Agency," 2019

Vaccine	Commercial name	PT (µg)	FHA (µg)	PRN (µg)	FIM2/3 (µg)	DT (IU)	TT (IU)	Al^{3+} (mg)
DTaP2	Pentavac®	25	25	-	-	≥30	≥40	0,3
Tdap3	Boostrix®	8	8	2,5	-	≥2	≥20	0,5
DTaP3 IPV-HBV	Infanrix Hexa®	25	25	8	-	≥30	≥40	0,5
Tdap5	Adacel®	2,5	5	3	5	≥2	≥20	0,33
DTaP-IPV	Pentacel®	20	20	3	5	15 Lf	5 Lf	0,3
DTaP-IPV-HB-Hib	Hexacima®	25	25	-	-	≥20	≥40	0,6

As the recent increase in whooping cough cases is seen mainly in older children and adolescents, this would suggest waning of childhood immunity. The efficiency and the memory induced by vaccination has become a subject of intense study. Studies of the baboon model suggest that similar antibody titers are elicited after vaccination with aP and wP vaccines. However, where the wP vaccine or previous infection prevented or shortened duration of *B. pertussis* colonization, the aP vaccine protected only from whooping cough symptoms, not colonization. Also, aP vaccinated animals readily transmitted the infection to co-housed animals. The cytokine profile of these animals shows that while a natural infection and wP

vaccination induce a Th1/Th17 response, the aP vaccine induces a Th2 polarized response (Warfel et al., 2014). This polarization has been confirmed in human studies by analysis of PBMN responses and cytokine profiles. Interestingly, these responses depend on the first vaccination, not on booster vaccination (Antunes et al., 2018; Bancroft et al., 2016). Also, humans vaccinated with aP vaccine had higher proportion of anti-pertussis antibodies of the IgG4 subclass. This tolerance-associated subclass is formed during Th2 response and is incapable of activating complement (van der Lee et al., 2018).

Another aspect of the recent increase of pertussis incidence is clearly evolution of the pathogen. As the acellular vaccine contains only a selection of antigens, this gives room for escape variants. For example, pertactin is an important antigen in many aP vaccines and anti-PRN antibodies have been proved important for antibody-mediated phagocytosis and correlated with protection against disease (Hellwig et al., 2003). Pertactin-negative (PRN$^-$) strains have been already documented in Europe (Bodilis and Guiso, 2013), USA (Martin et al., 2015) and Australia (Lam et al., 2014). In addition, comparison with pertactin-producing strains does not suggest reduced virulence (Bodilis and Guiso, 2013). Addition of another adhesin antigen, fimbriae, to aP vaccine has been shown to increase protection against PRN$^-$ strains in mice (Queenan et al., 2019). Analysis of antibody specificities after pertussis infection showed broader range of specificities in wP vaccinated humans compared to aP vaccinated individuals, although the antibody repertoire of aP vaccinated humans was not limited to vaccine components (Raeven et al., 2019).

All aforesaid evidence highlights the necessity of evaluation of vaccine efficacy. Measurement of phagocytosis is one of the many methods used for vaccine assessment.

1.3. Opsonophagocytic uptake and killing assays

Studying phagocytosis of opsonized or nonopsonized targets is a method widely used for a varying spectrum of reasons. Opsonophagocytosis assays are used to quantify phagocytosis under defined conditions. Under the collective term 'opsonophagocytic assay' are hidden two distinct types of tests. The 'opsonophagocytic uptake assay,' for the purpose of this thesis abbreviated OPA, is a method quantifying ingested particles. The other type, the 'opsonophagocytic killing assay' is used to measure the killing capacity of tested cells in specific conditions. Both types of assays find use in pathogen studies, such as in evaluating vaccines or comparing pathogen strains. But the applications are very wide – from studying

cellular processes in phagocytes, clearance of apoptotic or cancer cells to highlighting effects of mutations (Platt and Fineran, 2015). Due to the wide spectrum of applications, there are no universal optimal conditions. There are many variables in the setup of these assays and need to be tailored to specific needs. But in brief, generally a target particle is incubated with effector cells, an antibody source and complement source. Then uptake or killing of target particles is enumerated by one of available methods.

Opsonophagocytic assays have become widely used in serology of *Streptococcus pneumoniae*. Introduced in the publication of Romero-Steiner et al., it has become adapted by many (Romero-Steiner et al., 1997). The group of M. H. Nahm has standardized the assay and adapted it to be used as a high-throughput method allowing the simultaneous testing of antibodies against 4 serotypes of pneumococcus (Burton and Nahm, 2006). This standardized assay has been adapted and optimized for many different pathogens, such as *Neisseria meningitidis* (Humphries et al., 2015), *Salmonella enterica* (Ramachandran et al., 2016), or *Haemophilus influenzae* (Thomas et al., 2018).

The many variables of these assays are later described in more detail. A table containing an overview of the variables of published opsonophagocytosis assays can be found below (Table2).

Table 2 – overview of published literature employing opsonophagocytosis assays – Literature sorted by studied pathogen. Literature stating 'protocol' in the pathogen column is not research, but a published protocol. If relevant information was not provided by authors, panels are marked 'ns' (not specified). 'In serum' indicates that complement remains in serum. 'In blood' indicates that complement or antibodies are present in blood used for phagocytosis. Research of *Bordetella pertussis* is marked in bold.

PATHOGEN	PARTICLE	SERUM OR OTHER AB SOURCE	COMPLEMENT	PHAGOCYTE TYPE	UPTAKE OR KILLING	MOI (pathogen : particle)	ASSESSMENT METHOD	CITATION
Actinobacillus actinomycetemcomit-ans	Actinobacillus actinomycetemcomitans	Human IgG isolated from serum	Human hypogammaglobulin-emic serum 5%	Human isolated neutrophils	Killing	2:3-5	Plating	(Wilson et al., 1995)
Bordetella parapertussis	Bordetella parapertussis	Rabbit serum, purified antibodies	ns	Human isolated neutrophils	Uptake	1:30, 1:300	Confocal microscopy	(Gorgojo et al., 2012)
Bordetella parapertussis	Bordetella parapertussis	Mouse	In serum	Human isolated neutrophils	Uptake	1:50	Fluorescent microscopy	(Hayes et al., 2017)
Bordetella pertussis	**Bordetella pertussis**	**Human**	**IgG depleted human serum**	**Erythrocyte lysed blood, neutrophil population analysed**	**Uptake**	**ns**	**Flow cytometry**	(Aase et al., 2007)
Bordetella pertussis	**Zymosan particles**	**Human 50 %**	**In serum**	**THP-1 monocyte-like cell line**	**Uptake**	**10 µl/10^5 cells, 100 µl/10^6 cells**	**Flow cytometry**	(Hasan et al., 2019)
Bordetella pertussis	**Heat killed B. pertussis**	**Rabbit IgG isolated from serum**	**ns**	**Mouse leucocytes from blood**	**Uptake**	**ns**	**Flow cytometry**	(Hellwig et al., 2001)
Bordetella pertussis	**Bordetella pertussis**	**Human**	**In serum**	**Human isolated neutrophils**	**Uptake**	**1:70**	**Flow cytometry**	(Hellwig et al., 2003)
Bordetella pertussis	**Bordetella pertussis**	**Human**	**ns**	**Human isolated neutrophils**	**Uptake**	**1:10**	**Flow cytometry**	(Hovingh et al., 2018)
Bordetella pertussis	**Bordetella pertussis**	**Human isolated IgG**	**ns**	**Human isolated neutrophils**	**Uptake**	**1:50**	**Flow cytometry**	(Lamberti et al., 2008)

Bordetella pertussis	Bordetella pertussis	Human isolated IgG	ns	Human monocyte-derived macrophages	Uptake	1:20 - 1:50	Plating, flow cytometry, confocal microscopy	(Lamberti et al., 2010)
Bordetella pertussis	Bordetella pertussis	ns	ns	A549 human lung epithelial cell line	Uptake	1:150	Plating, fluorescent microscopy, confocal microscopy	(Lamberti et al., 2013)
Bordetella pertussis	Bordetella pertussis	Human	In serum	Human isolated neutrophils	Uptake	1:10	Flow cytometry	(Lenz et al., 2000)
Bordetella pertussis	Bordetella pertussis	Human	ns	Isolated human neutrophils	Uptake	ns	ns	(Mobberley-Schuman et al., 2003)
Bordetella pertussis	Bordetella pertussis	ns	ns	Isolated human neutrophils	Uptake	ns	ns	(Mobberley-Schuman and Weiss, 2005)
Bordetella pertussis	Staphylococcus pneumoniae	Human 0,03–0,0002 %	Baby rabbit	NB-4 human lekemia cell line	Killing	ns	Plating	(Prior et al., 2006)
Bordetella pertussis	Bordetella pertussis	Human isolated IgG and IgA, rabbit isolated IgG	ns	Human isolated neutrophils	Killing and uptake	1:70	Plating and flow cytometry	(Rodriguez et al., 2001)
Bordetella pertussis	Bordetella pertussis, Escherichia coli	Human serum and IgG	Added, but not specified or not added	Human isolated neutrophils	Killing and uptake	1:60	Microscopy, flow cytometry, plating	(Steed et al., 1992, 1991)
Bordetella pertussis	Bordetella pertussis	Human	ns	THP-1 monocyte-like cell line	Uptake	1:100	Microscopy	(Stefanelli et al., 2002)
Bordetella pertussis	Bordetella pertussis	mAbs, human serum	In human serum, guinea pig	Human isolated neutrophils	Uptake	1:6	Fluorescent microscopy	(Weingart et al., 2000; Weingart et al., 2000; Weingart and Weiss, 2000)

Candida albicans	Candida albicans	ns	ns	Human isolated neutrophils, mouse peritoneal cells	Killing and uptake	1:1 killing, 1:4 uptake	Flow cytometry, microscopy, plating, percoll separation and radiometric viability assay	(van Spriel et al., 1999)
Chlamydia trachomatis	Formalin fixed C. trachomatis	Rabbit, mouse, human 0,01-10 %	In serum	PLB-985 human lekemia cell line	Uptake	1:2-40:1	Flow cytometry	(Grasse et al., 2018)
Escherichia coli	Escherichia coli	Rat and human	2-7 % Human	HL-60 human lekemia cell line	Killing	600:1	Plating	(Abbanat et al., 2017)
Haemophilus influenzae	Haemophilus influenzae	Human and mouse	IgG depleted human plasma	HL-60 human lekemia cell line	Uptake	1:50	Flow cytometry	(Thomas et al., 2018)
Haemophilus influenzae	Haemophilus influenzae	Human serum and IVIG, guinea pig	Guinea pig, IgG depleted human serum	HL-60 human lekemia cell line	Killing	100:1	Plating	(Winter and Barenkamp, 2016, 2014, 2003)
Haemophilus influenzae and Streptococcus pneumoniae	Haemophilus influenzae and Streptococcus pneumoniae	ns	Serum from immunodeficient mice	Mouse isolated peritoneal neutrophils	Killing	1000:1	Plating	(Lysenko et al., 2005)
Klebsiella pneumoniae	Klebsiella pneumoniae	Human 5 %	In serum	Human isolated neutrophils	Killing and uptake	1:10	Plating and fluorescent microscopy	(Kobayashi et al., 2016)
Neisseria gonorrhoeae	Neisseria gonorrhoeae	Human	Human serum depleted of C8	Human isolated neutrophils	Killing	1,25:0,8	Plating	(Kim et al., 1992)
Neisseria meningitidis	Neisseria meningitidis	Human	Human serum	human isolated neutrophils, isolation method unspecified	Uptake	2:50	Flow cytometry	(Aase et al., 2003)
Neisseria meningitidis	Neisseria menigitidis	Whole human blood	In blood	Human whole blood	Killing	ns	Plating	(Granoff, 2009)

Neisseria meningitidis	Live or sodium azide and phenylmethylsulfo-nyl fluoride killed N. meningitidis	Human, mouse	IgG depleted human plasma	HL-60 human lekemia cell line	Killing and uptake	40:1 killing, 1:50 uptake	Plating and flow cytometry	(Humphries et al., 2015)
None	Polystyrene beads	Purified antibodies	ns	Human isolated neutrophils	Uptake	1:10	Microscopy	(Golay et al., 2019)
None	Cryptococcus neoformans	Purified antibodies	ns	Mouse peritoneal macrophages, J774 mouse macrophage cell line	Uptake	1:1-1:2	Microscopy	(Hawk et al., 2019)
None	Fixed Escherichia coli and Staphylococcus aureus (pHrodo bioparticles), latex beads, zymosan particles	Mouse IgG, Human serum	In serum	Mouse bone-marrow derived macrophages, Human induced pluripotent stem cell derived macrophages, RAW264.7 mouse macrophage cell line, THP-1 and BV-2 mouse microglial cell line	Uptake	1 µg to 1mg/ml	Fluorescent microscopy, confocal microscopy	(Kapellos et al., 2016)
None	Latex beads	Rabbit IgG	Human serum	Human isolated neutrophils	Uptake	1:8	Flow cytometry	(Kobayashi et al., 2002)
None	Heat-killed Escherichia coli, sheep erythrocytes, zymosan	ns	ns	Human monocyte derived macrophages	Uptake	20 µl/10^6 cells	Flow cytometry	(Mendoza-Coronel and Ortega, 2017)
None	Zymosan particles	Human 10 %	In serum	Human neutrophils: isolated or spontaneously sedimented	Uptake	1:10	Microscopy	(Mosca and Forte, 2016)
None	Sheep erythrocytes	Rabbit IgG or IgM isolated from serum	Human C5 deficient serum	RAW264.7 and mouse bone-marrow macrophages	Uptake	ns	Microscopy	(Naik et al., 2019)
Pseudomonas aeruginosa	Pseudomonas aeruginosa	Added 10 %, but not specified	In serum	Mouse isolated bone-marrow neutrophils and human isolated neutrophils	Killing and uptake	1:3	Plating and confocal microscopy	(Monfregola et al., 2012)
Salmonella enterica	Salmonella enterica	Mouse	Baby rabbit	HL-60 human lekemia cell line	Killing	100:1	Plating	(Ramachandran et al., 2016)

Salmonella enterica	Salmonella enterica	Mouse	In serum	J774 mouse macrophage cell line	Killing	1:1	Plating	(Simon et al., 2011)
Salmonella enterica	Salmonella enterica	Mouse	In serum	J774 mouse macrophage cell line	Uptake	10:1	Plating	(Tennant et al., 2011)
Staphylocccus epidermidis	Staphylocccus epidermidis	Human	Human, in serum	Human isolated neutrophils	Uptake	1:4	Flow cytometry and fluorescent microscopy	(Heinzelmann et al., 1997)
Staphylococcus aureus	Live and heat-killed S. aureus	Human 4 %	Human, in serum	Human isolated neutrophils and monocytes	Killing and uptake	25:1 killing, 1:34 and 1:3,4 neutrophil uptake, 10:1 monocyte uptake	Plating, flow cytometry and fluorescent microscopy	(Collins et al., 2002)
Staphylococcus aureus	Lactococcus lactis	ns	Factor B depleted serum	Neutrophils, species ns	Killing	1:1	Plating	(Hair et al., 2013)
Staphylococcus aureus	Staphylococcus aureus	Human	In serum	Human isolated neutrophils	Uptake	1:1 and 1:10	Microscopy	(Lu et al., 2014)
Staphylococcus aureus	Lactococcus lactis	Human	In serum	Human isolated neutrophils	Killing	ns	Plating	(Sharp et al., 2012)
Staphylococcus aureus and Escherichia coli	Staphylococcus aureus and Escherichia coli	Human 30 %	In serum	Human isolated neutrophils	Uptake	1:500	Atomic force microscopy	(Pleskova et al., 2018)
Staphylococcus aureus and Staphylococcus epidermidis	Staphylococcus aureus and Staphylococcus epidermidis	Human	In serum	Human isolated neutrophils	Killing	1:2, 1:1	Fluorescence measurements	(van Grinsven et al., 2018)
Staphylococcus epidermidis	Staphylococcus epidermidis	Human 20 %	In serum	Human isolated neutrophils	Killing	1:1	Plating	(Kristian et al., 2008)
Streptococcus	Streptococcus gordonii	Human (in blood)	In blood	Human whole blood and isolated neutrophils	Killing	1:1	Plating	(Itzek et al., 2017)
Streptococcus agalactiae	Streptococcus agalactiae	Rabbit and mouse 10 %	Baby rabbit 10 %	HL-60 human lekemia cell line	killing and uptake	25:1-40:1 killing, 1:10 uptake	plating and flow cytometry	(Fabbrini et al., 2012)

Streptococcus agalactiae	Streptococcus agalactiae	Rabbit	Mouse serum	Human leukocytes	Killing	1:2	Plating	(Wessels et al., 1995)
Streptococcus pneumoniae	Streptococcus pneumoniae	IVIG	Added, but not specified	Mouse alveolar, peritoneal and J774 cell line macrophages	Killing	100:1	Plating	(Bangert et al., 2012)
Streptococcus pneumoniae	Streptococcus. pneumoniae	Human 0,1-30 %	Baby rabbit	HL-60 human lekemia cell line	K illing	100:1, 200:1, 400:1	Plating	(Burton and Nahm, 2012, 2006)
Streptococcus pneumoniae	Streptococcus pneumoniae	Human	Baby rabbit 66 %, Human in serum 10 %	Human isolated neutrophils	Killing and uptake	100:1 killing, 1:2 uptake	Plating, flow cytometry	(Dalia et al., 2010)
Streptococcus pneumoniae	Streptococcus pneumoniae	ns	Baby rabbit	Human isolated neutrophils	Killing	1000:1	Viable counts, method ns	(Davis et al., 2008)
Streptococcus pneumoniae	Streptococcus pneumoniae	Human	ns	HL-60 human lekemia cell line	Killing	400:1	Plating	(Kim et al., 2003)
Streptococcus pneumoniae	Streptococcus pneumoniae	ns	Added, but not specified	Mouse isolated peritoneal neutrophils and monocytes	Killing	1000:1	Plating	(Lysenko et al., 2007)
Streptococcus pneumoniae	Streptococcus pneumoniae	Human	Rabbit	HL-60 human lekemia cell line	Killing	1:40	Plating	(Nahm et al., 2000)
Streptococcus pneumoniae	Streptococcus pneumoniae	Human 12,5 % - 0,01 %	Baby rabbit	HL-60 human lekemia cell line, human isolated neutrophils	Killing	400:1 HL-60, 500:1 neutrophils	Plating	(Romero-Steiner et al., 1997)
Streptococcus pneumoniae	Streptococcus pneumoniae	Human	Added, but not specified	Human isolated neutrophils	Killing	500:1	Plating	(Simell et al., 2011)
Streptococcus pneumoniae	Streptococcus pneumoniae	Purified antibodies added, but not specified	Baby rabbit	Human isolated neutrophils, Mouse isolated peritoneal neutrophils	Killing and uptake	100:1	Plating and flow cytometry	(Standish and Weiser, 2009)
Streptococcus pneumoniae	Streptococcus pneumoniae	mABs	Mouse	Mouse isolated peripheral blood neutrophils, J774 mouse macrophage cell line	Killing and uptake	10000:1, 500:1, 400:1, 50:1, 5:1, 1:2, 1:10	Plating and flow cytometry	(Tian et al., 2009)
Streptococcus pyogenes	Streptococcus, various strains	Human	In serum	Human isolated neutrophils	Killing and uptake	1:5	Plating and microscopy	(Lei et al., 2001)

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Streptococcus pyogenes	Streptococcus pyogenes	Human	In serum	Human isolated neutrophils, whole blood	Killing	1000:1 neutrophils, 1000/0,25 ml blood	Plating	(Nilsson et al., 2005)
Streptococcus pyogenes	Streptococcus pyogenes	Rabbit, human IVIG	Added, but not specified	HL-60 human lekemia cell line, whole blood	Killing	100000:2, 10000:2, 1000:2	Plating	(Salehi et al., 2018)
Streptococcus pyogenes	Streptococcus pyogenes	Human plasma	In plasma or blood	Human isolated neutrophils and whole blood	Killing	1:2,5 neutrophils, 10^4CFU/300µL whole blood	Plating	(Zinkernagel et al., 2008)
Various pathogens	HIV protein, influenza protein, Ebola virus protein, tetanus toxoid-coupled fluorescent beads	Human	In serum	Human leucocytes aquired by erythrocyte lysis and isolated neutrophils	Uptake	ns	Flow cytometry	(Karsten et al., 2019)
Various pathogens	Various live pathogens	In blood	In blood	Human whole blood	Killing	$(10^3$–10^5CFU/100µL blood)	Plating and flow cytometry	(van der Maten et al., 2017)
Yersinia pestis	Yersinisa pestis	ns	ns	Mouse peritoneal macrophages	Killing and uptake	1:3, 1:10	Fluorescent microscopy	(VanCleave et al., 2017)
Protocol	Bacteria of interest	ns	10-30 % dilution recommended	Human isolated neutrophils and HL-60 human lekemia cell line	Killing	1:1, but testing advised in interval of 1:1 to 1:250	Plating	(Dwyer and Gadjeva, 2014)
Protocol	pHrodo conjugated bioparticles	ns	In blood or in situ	Whole blood or mouse peritoneal neutrophils in vivo	Uptake	ns	Flow cytometry	(Fine et al., 2017)
Protocol	Heat-killed Candida albicans, use of bacteria is mentioned	Human	In serum	Mouse bone-marrow monocytes, peripheral blood neutrophils, human leukocytes	Uptake	ns	Flow cytometry	(Maini et al., 2018)
Protocol	Sheep erythrocytes and bacteria of interest, Mycobacteria as an example.	ns	ns	Mouse bone-marrow macrophages, J774, RAW264.7,	Uptake	1:10 to 1:50 for sheep erythrocytes, 1:5-1:500 for mycobacteria	Fluorescent microscopy and flow cytometry	(Platt and Fineran, 2015)

Protocol	Bacteria, prefer fixed over live	ns	Baby rabbit, Human hypogammaglobulin emic serum	Human blood lysis acquired neutrophils, HL-60	Uptake	1:40, testing recommended	Flow cytometry	(Plested and Coull, 2003)
Protocol	Latex beads	FBS or IgG	ns	RAW264.7	Uptake	1:10-1:100	Flow cytometry and fluorescent microscopy	(Sharma et al., 2014)

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1.3.1. Cells

The first variable addressed would be the type of phagocytic cell used, since choice of cell types may influence other variables. There is a difference of opinion on the optimal cell type. Effector cell should be determined by biological relevance of the model and reproducibility of results. When a uniform phagocyte population is desirable, one option is to use a cell line. Though this yields more reproducible results (Nahm et al., 2000), there is a need for tissue-culture facility. Also, differentiation into phagocytes where the percentage of actually differentiated cells is high may be technically difficult to achieve (Plested and Coull, 2003). Biological relevance of using immortalized lines is also disputable. Among the most used human derived cell lines is HL-60. Other human options are THP1 or U937. Among murine cell lines, the most commonly used is J774, less often used are RAW264.7 cells.

Table 3 lists the available cell line options and their differentiation requirements, also the options available when using primary cells.

The other option, when a uniform population of cells is preferred, are isolated primary cells. Preferred are human or mouse neutrophils, monocyte derived macrophages and, for mice, peritoneal macrophages are also an option (Platt and Fineran, 2015). More phagocytic cell types available are listed in Table 3. Isolated neutrophils of mouse or human origin seem to be the cell of choice for many researchers, based on data from Table 3. Although neutrophils are short lived cells, they express Fc receptors and complement receptors in high levels, their response is rapid and they are well equipped for bacterial killing (Karsten et al., 2019).

Where biological relevance is favored before population uniformity, a mixed population of leukocytes, or even whole blood may be used. If populations are analyzed separately, as by flow cytometry, both high biological relevance and data acquisition for each individual population can be preserved. Examples of using human and mouse leucocytes can be found in the work of Karsten et al., or in the article of Hellwig et al. (Hellwig et al., 2001; Karsten et al., 2019). Using whole blood has a drawback for studying serum samples. Since whole blood is unmanipulated, it already contains antibodies and complement intrinsically. This may conceal sample effect, especially when using targets opsonized by negative samples or blood from immunized donors.

Table 3 – Most commonly used phagocytic cells and their culture conditions. Adapted from Platt and Fineran, 2015.

Cell type	Species of origin	Primary or immortalized	Differentiation conditions
HL-60	Human	Immortalized	dimethylformamide
THP1	Human	Immortalized	Phorbol ester
U937	Human	Immortalized	Phorbol ester
J774	Mouse	Immortalized	Culture in complete medium
RAW264.7	Mouse	Immortalized	Culture in complete medium
Monocyte derived macrophage	Human	Primary, differentiated	Human M-CSF
Monocyte derived dendritic cell	Human	Primary, differentiated	Human GM-CSF
Monocyte	Human	Primary	Culture in complete medium
Neutrophil	Human	Primary	Not culturable
Peritoneal macrophage	Mouse	Primary	Culture in complete medium
Bone-marrow derived macrophage	Mouse	Primary, differentiated	M-CSF
Bone-marrow derived dendritic cell	Mouse	Primary, differentiated	GM-CSF
Neutrophil	Mouse	Primary	Not culturable

Neutrophils were chosen as the effector cells for assays performed in this thesis. They are a population that has been shown to be relevant in antibody-dependent clearance of *B. pertussis* in the mouse (Andreasen and Carbonetti, 2009). Also, many of the virulence factors of *B. pertussis* are aimed at neutrophils. Pertussis toxin delays early neutrophil influx into lungs (Kirimanjeswara et al., 2005), ACT has been shown to have many effects on neutrophils – blocking chemotaxis, phagocytosis, oxidative burst, NET formation and apoptosis (Cerny et al., 2017; Confer and Eaton, 1982; Eby et al., 2014; Hasan et al., 2019; Weingart and Weiss, 2000). Although the role of neutrophils in *Bordetella pertussis* infection in humans is not yet completely understood, it is clear that they are a relevant population to study. Also, there are many assays performed with this type of cell. Most opsonophagocytic assays with neutrophils use isolated cells, I opted to perform the opsonophagocytic assay with isolated neutrophils.

Mouse neutrophils were my first choice, due to availability of murine sera. Human neutrophils were second option, as they are utilized in the majority of assays.

1.3.2. Neutrophil isolation method

As seen in Table 2, isolation of neutrophils is used in a large number of opsonophagocytic assays. Most of the neutrophils are isolated on some sort of gradient by centrifugation. Gradients vary in the reagents and densities used. Concerning mouse neutrophil isolation, Swamydas et al. provide six protocols for isolating mouse neutrophils. Bone-marrow is presented as the best source for obtaining neutrophils, followed by peripheral blood and peritoneal exudate. Neutrophils from these sources are isolated on a discontinuous gradient, created by Histopaque densities 1,119/1,077 g/ml or 40 %/70 % Percoll in PBS. To harvest a relevant number of neutrophils from peritoneum, they are stimulated by caseine injection prior to isolation. This priming has been shown to elicit an activated phenotype of the neutrophils, which may influence phagocytosis. The authors recommend the bone-marrow, as it yields the highest number of neutrophils per mouse (Swamydas et al., 2015). Also, bone-marrow derived neutrophils have been shown to be functionally equivalent to circulating neutrophils in many markers and activity assays. Unfortunately phagocytosis was not one of the studied parameters (Boxio et al., 2004). Maini and colleagues introduce a protocol for immunomagnetic separation, which is possibly gentler to the isolated neutrophils (Maini et al., 2018). Tian et al. (2019) isolated neutrophils from mouse blood, and some groups opted for the casein-elicited peritoneal exudate isolation. Fine et al. (2017) used peritoneal neutrophils and to conserve biological context of cell populations, the opsonophagocytosis was performed in vivo (Lysenko et al., 2005; Monfregola et al., 2012; Tian et al., 2009).

For obtaining isolated human neutrophils, heparinized peripheral blood is used. Sometimes, EDTA or citrate are used as anticoagulant. Even more than in assays performed with murine cells, there is a variability in the gradients employed for isolation. Many gradients are commercially available and do not require any preparation ahead of the assay. Most often, Ficoll gradient is used. Other encountered options include Percoll gradients (Kristian et al., 2008), Histopaque (Hellwig et al., 2003). Some protocols use PolymorphPrep (Axis-Shield), (Zinkernagel et al., 2008). Many researchers choose to precede the gradient centrifugation with a sedimentation step to remove excess erythrocytes. Dextran is used most often, followed by Ficoll (Quach and Ferrante, 2017).

Comparisons of the available methods provide more information by examining the properties of isolated neutrophils. Pre-separation of leukocytes by dextran or Ficoll sedimentation has

been shown to cause activation of the isolated neutrophils. The mechanism seems to be monocyte-dependent (Quach and Ferrante, 2017). Activation may increase neutrophil phagocytic functions, but also in control samples. As this reduces differences between samples and controls, generally less activated neutrophils are favored (Mosca and Forte, 2016). The effects of different separation methods have also been analyzed. Haslett et al. (1985) describe three methods of neutrophil isolation, each starting with a dextran sedimentation step. They compare i) a Ficoll based gradient technique, ii) a pasma-Percoll gradient technique and iii) an erythrocyte lysis technique. The Ficoll-isolated neutrophils showed higher priming after isolation than the other two methods, assessed by change of shape and chemotaxis. They also show little response to further stimulation by LPS (when ROS production and enzymes secretion evaluated). Methods ii) and iii) were seen as equivalent. The erythrocyte lysis method yields a neutrophil purity of 80 % compared to 99 % obtained by the other two methods. As all these techniques were preceded by dextran sedimentation, the role of dextran in activation was not addressed. An effect of NH_4Cl used for erythrocyte lysis was also shown to induce some signs of activation when compared to lysis with hypotonic saline (Haslett et al., 1985). Another comparison included iv) Percoll-in-PBS discontinuous gradient, v) Ficoll gradient and vi) spontaneous sedimentation. Chemotaxis, phagocytosis of zymosan particles, digestion (nitroblue tetrazolium reduction test) and change of shape of the obtained neutrophils were examined. The cell yields of the methods were comparable, only the spontaneous sedimentation produced lower purity neutrophils (77 %). The chemotaxis was similar in methods iv) and v), spontaneous sedimentation gave lower chemotactic values. Cells obtained by spontaneous sedimentation were the only population which showed a difference in chemotaxis between stimulation with LPS and stimulation with LPS and serum. The rates of zymosan ingestion were similar in all isolated cells. Although the spontaneously sedimented cells had a slightly lower value of NBT reduction compared to the other two methods, they displayed the greatest difference in values compared to control. Methods iv) and v) yielded a significant portion of neutrophils with shape changes, around 35 % for Percoll gradient and 55-60 % for Ficoll gradient. No such changes were observed in the spontaneously sedimented cells (Mosca and Forte, 2016).

An alternative to isolation that is applied in some assays is use of a mixed leukocyte population. The populations are most often analyzed separately by gating the target populations in flow cytometry software (Haslett et al., 1985; Mosca and Forte, 2016). Ammonium chloride solution or ammonium-chloride-potassium (ACK) are common lysis buffers. Leukocytes prepared by

such lysis are used by several groups (Aase et al., 2007; Karsten et al., 2019; Maini et al., 2018; Plested and Coull, 2003; Wessels et al., 1995). Hypotonic NaCl lysis is also a valid method, used by Haslett et al., who also included a comparison with ammonium chloride lysis. NaCl induced less activation of the neutrophils than ammonium chloride. Another comparison focused on ACK and commercially available lysis buffers and their suitability for flow cytometry. They show marked differences in the scatter of populations separated by flow cytometry, also in fluorescence levels of each sample. Lysis by ACK buffer generated high amount of debris and showed a high fluorescence of isotype controls but provided good separation of populations. The authors demonstrated that lysis method may have an impact on T-cell populations, such as $CD4^+/CD8^+$ ratio, and on granulocytes, with some of the commercial lysis buffers generating a granulocyte population compressed in the FSC gate (Bossuyt et al., 1997).

A completely different approach was chosen for an in-vivo assay. Mouse peritoneal neutrophils were chosen as phagocytic cells and to conserve biological context of cell populations, the opsonophagocytosis was performed in vivo. Fluorescently labeled inactivated microorganisms were injected into the mouse peritoneum and harvested the exudate. The sample contained mixed leukocytes with ingested bioparticles and the population of choice, the neutrophils, was separated by gating (Fine et al., 2017).

Whole blood does not require any preparation aside from anticoagulant addition, but the presence of erythrocytes complicates the evaluation by flow cytometry or microscopy. Whole blood samples are thus rid of erythrocytes after the opsonophagocytosis has been allowed to take place (Fine et al., 2017).

In most opsonophagocytic assays, phagocytic cells and tested serum samples are from the same species. However, there are valid reasons to choose differently. For example, complexity of isolation, or low cell yield may be reasons to rethink the donor species. For instance, in a *Streptococcus pneumoniae* opsonophagocytic assay human IVIG was used as opsonin and mouse cells were used as effector cells (Bangert et al., 2012). More commonly, the situation is reversed and sera from model animals, most often mouse or rabbit, are used to opsonize targets for human cells. In one assay mouse and rabbit sera were used together with the human HL-60 cell line, others used mouse sera together with human isolated neutrophils (Fabbrini et al., 2012; Hayes et al., 2017).

1.1.1. Particles

For measuring phagocytosis of pathogens, the concerned microorganism is most often used. For killing assays, live microorganisms are necessary. In sporadic cases a different microorganism than is studied is used as target particle (Hair et al., 2013). For uptake assays, killed microorganisms are sometimes preferred for safety reasons or convenience of storage (Maini et al., 2018), but as seen in Table 2, use of live microorganisms still predominates. For used live *Staphylococcus aureus* for a killing assay and heat-killed *S. aureus* for an uptake assay (Collins et al., 2002).

Other particles available are erythrocytes, labeled zymosan or *E. coli* bioparticles or synthetic particles, such as beads from different materials. Beads labeled with fluorochromes are particularly useful in flow cytometry analyzed assays (Platt and Fineran, 2015; Sharma et al., 2014). Examples of such particle use can be found in protocols of Fine et al. or Sharma et al. (Fine et al., 2017; Sharma et al., 2014). It is important to note that opsonization of synthetic particles, or any particles different from the pathogen studied, is antigen-unspecific.

1.1.2. Multiplicity of infection

The ratio of effector cells (phagocytes) to particles is called 'multiplicity of infection,' abbreviated MOI. A correctly chosen MOI will maximize differences among tested conditions. The MOI ratios vary greatly among opsonophagocytic assays (Table 2) and is a variable that requires optimization for each specific assay. In general, assays using cell lines such as HL-60 employ MOI between 500:1 to 10:1, as in the article by Burton and Nahm from 2006. MOI of assays using isolated neutrophils varies more, generally between 100:1 to 1:500, but the majority of is set close to 1:10 ratio, as in Kobayashi et al. from 2016. When using leukocytes or whole blood, precise determination of MOI is difficult without a previous cell count by flow cytometry. In those cases, authors generally indicate the MOI by volumes, for example Zinkernagel et al. add 10^4 CFU/300µL whole blood (Zinkernagel et al., 2008). Interestingly, some MOI encountered in literature are even more unequal: Lysenko et al. in both articles use a MOI of 1 000:1, Salehi et al. even tested MOI up to 10^5:1 (Lysenko et al., 2007, 2005; Salehi et al., 2018).

1.1.3. Antibody source

To elicit maximum phagocytosis, an antibody and a complement source are used for opsonization. In some cases, the role of each is studied individually, the target particle being opsonized by antibody only or by complement only (Naik et al., 2019). In pathogen studies, mostly human serum samples are the source of antibody, less commonly serum acquired from

a mouse model. In some cases, IVIG is used as antibody source (Bangert et al., 2012; Salehi et al., 2018; Winter and Barenkamp, 2003). Mouse or other animal models give the advantage of obtaining serum from a naïve individual. Cross-reactive or even specific antibodies are present in the serum of many humans, from vaccination or encountering the pathogen, even if no infection was noted (Lu et al., 2014). Routine vaccination is administered in prevention of many pathogens and in case of *Bordetella pertussis* this is administered in infancy. IgG depletion could provide a possible negative control (Brookes et al., 2013), but this serum is depleted of all, even unspecific antibodies.

Monoclonal antibodies are also used in opsonophagocytic assays. mAbs are often tested in opsonophagocytic assays as therapeutic antibodies, with specificities against pathogens or cancer cells (Platt and Fineran, 2015; Tian et al., 2009).

1.1.4. Complement source

Complement opsonization also contributes to opsonophagocytic uptake and killing. In some assay setups, there is no need to add extrinsic complement. When using whole blood as effector cell population, it serves as complement source (Granoff, 2009). Also, tested serum samples naturally contain complement. Many research groups do not add any complement externally and rely on the complement in serum samples. These are marked in Table 2 as 'in serum.' The downside of this is interdonor variability. For samples from inbred mice, this may not be large, but greater differences can be observed in humans. In cases where standardization is necessary, an external complement source is used and serum samples are heat-inactivated before use in assays (Burton and Nahm, 2006).

An ideal external complement source contains a minimum of possibly cross-reactive antibodies. Sometimes 'normal human serum,' a pool of sera from healthy individuals, is used as complement source. This circumvents the interdonor variability, but possible antibody cross-reactivity remains (Brookes et al., 2013). As external source of complement, serum of agammaglobulinemic patients can be used (Plested and Coull, 2003; Wilson et al., 1995). Agammaglobulinemic patients are rare, and often receive therapy for their condition, IVIG, thus are not suited as a regular source of complement (Brookes et al., 2013). Baby rabbit complement is used as a complement source in many assays, such as in the standardized pneumococcal opsonophagocytosis assays developed by Romero-Steiner et al. (1997) and expanded by Burton and Nahm, (2006). Baby rabbit complement provides good standardization, but also high levels of nonspecific killing (Santos et al., 2001). Other sources include naïve mouse serum, but when taken from most laboratory strains of mice this has very

low complement activity compared to human, rabbit or even wild-population mouse complement (Bergman et al., 2000; Ong and Mattes, 1989). In some cases, low complement activity can be considered desirable, possibly highlighting effector cell functions, but in many assays, complement-mediated lysis is part of assessment of antibody function. As a reliable and reproducible source of complement, IgG depleted serum has been developed by the group of A. Gorringe (Brookes et al., 2013). IgG depleted human serum has been used in further assays of this research group, for example in the work of Humphries et al., Thomas et al., or others (Humphries et al., 2015; Thomas et al., 2018).

Unfortunately, many authors do not specify if any external complement source was used. Some only specify that 'complement source' was added, but not any specific details, like species or concentration.

For ease of assay feasibility, complement was retained in serum samples tested in this thesis. Paired heat-inactivated samples were tested side by side with no other complement source, to evaluate complement role. In the murine serum samples intrinsic complement activity was expected to be low. In the human serum samples, high complement activity was expected.

1.1.5. Serum concentration

Sera are used in assays during the opsonization steps in varying concentrations. Generally, the concentrations range from 0,01 % to 30 %. The most common value is 10 % serum. In some cases, as high as 50 % serum is used (Hasan et al., 2019). Such a high concentration is usually used for nonbiological particles or killed microorganisms.

1.1.6. Assessment methods for OPK

The evaluation method is in many ways determined by previously mentioned variables. In the case of killing assays, the only generally used method of evaluation is determining the viability count by plating. The reaction volume is diluted and plated onto agar (Abbanat et al., 2017). Optionally, lysis of phagocytes may be included before plating (Collins et al., 2002; Kobayashi et al., 2016). The plating method is reliable but has several drawbacks. First, plating is a time-consuming process, limiting the number of samples processed by one laboratory worker per day. Also, it is necessarily followed by incubation, which is around 5 days for *Bordetella pertussis*, and CFU counting. Colony counting software can be used to hasten the procedure. Last, since different amount of killing or uptake is expected from individual samples, it is required to perform and plate several dilutions, to obtain a countable number of CFUs. The difference in CFU between controls (without added antibodies, complement or phagocytes) and samples is usually expressed as a percentage (van der Maten et al., 2017). Percentage of

surviving or percentage of killed bacteria can be listed. When phagocyte-specific killing is studied, number of CFU in a sample with phagocytes is compared to a number of CFU in an identical sample without phagocytes. According to protocol of Dwyer and Gadjeva (2014), a 40 % difference between these values can be considered significant killing. If different serum concentrations are tested, 'opsonization titer' can be calculated as the serum dilution, that kills 50 % bacteria (Burton and Nahm, 2006).

1.3.3. Assessment methods for OPA

For the evaluation of uptake assays, more options are available and used. Plating is also an option in this case (Itzek et al., 2017; Tennant et al., 2011). When uptake and not killing is analyzed by plating method, washing steps to remove extracellular bacteria are employed, or extracellular bacteria can be killed by addition of antibiotics, usually gentamicin. In a different study however, gentamicin has been shown to negatively influence the viability of intracellular bacteria as well (VanCleave et al., 2017). Microscopy is a method highly favored by researchers, as shown in Table 2. Assessment by microscopy provides a sufficient resolution, for example to distinguish extracellular particles from intracellular. The disadvantage is that quantifying objects by microscopy is time-consuming. Counting hundreds of cells is required to provide significant results (Platt and Fineran, 2015). If erythrocytes are used as target particle, extracellular erythrocytes can be lysed by hypotonic solution before counting (Naik et al., 2019). Fluorescent microscopy gives the advantage of staining of cells or particles. Mainly, fluorescent staining is used for differentiation of extracellular from intracellular particles. There are several approaches to discerning localization of particles. Quenchers of fluorochromes can be applied to extracellular particles. This approach is simple but causes loss of information on extracellular attachment, which is also an important readout in some assays (Collins et al., 2002; Heinzelmann et al., 1997; C. L. Weingart et al., 2000). A compound or antibody labeled with a second fluorochrome can be applied to extracellular bacteria. The double-labeled bacteria can then be subtracted from the total counts. This method allows to quantify ingested and adherent bacteria separately. The second stain for extracellular particles can detect the particle itself, or the opsonins on the particle surface. Confocal microscopy is sometimes used (Gorgojo et al., 2012; Monfregola et al., 2012). The output of microscopy analysis is percentage of cells containing a particle out of all counted cells (Golay et al., 2019; Gorgojo et al., 2012; Kobayashi et al., 2016).

Flow cytometry is also often applied for sample analysis. Large samples can be quickly and easily quantified by this method. On the downside, differential staining as described above for

fluorescent microscopy is required for discrimination of attached particles from ingested (Platt and Fineran, 2015). Even so, many researchers opt to use only single-stained particles and count all positive events as ingested (Dalia et al., 2010; Humphries et al., 2015). Collins et al. demonstrated that the amount of extracellular particles was negligible, but Hellwig et al reported about 16,5 % extracellular bacteria (Collins et al., 2002; Hellwig et al., 2001). As with fluorescent microscopy samples, the extracellular stain can detect the particles (Lamberti et al., 2008) or opsonins on the particles (Hellwig et al., 2003, 2001; Lamberti et al., 2008). Alternatively, detection can be focused on respiratory burst of neutrophils after particle ingestion. Such technique was used by Aase et al. in their articles. They labeled the phagocytes with dihydrorhodamine 123, which is nonfluorescent until converted to rhodamine 123 by respiratory burst (Aase et al., 2007, 2003). In samples analyzed by flow cytometry, the level of phagocytosis can be expressed in multiple ways. First, as in microscopy, the percentages of cells containing particles from all cells are calculated. For estimation of the amount of ingested particles, mean fluorescence intensity (MFI) is used (Fabbrini et al., 2012). Hovingh et al. used MFI to calculate arbitrary opsonophagocytic indices by dividing the MFI obtained from phagocytosis of sample-opsonized particles by the MFI obtained from phagocytosis of nonopsonized targets (Hovingh et al., 2018). Karsten and colleagues calculated a phagoscore combining both percentage and MFI readouts. This was determined as [(geometric mean of fluorescence intensity of positive cells) × (% positive cells)]/10000 (Karsten et al., 2019).

Some more exotic methods were used for assay evaluation such as radiometric viability assay (van Spriel et al., 1999) or overall fluorescence measurement (van Grinsven et al., 2018). Pleskova et al. even use atomic force microscopy, which allowed them to study the dynamics of phagocytosis in real time (Pleskova et al., 2018).

A combination of methods is used by researchers to correlate data acquired from each method. Combining microscopy analysis with flow cytometry is widely used, for example in the work of Heinzelmann et al. (1997) and is recommended in the methodology chapter by Platt and Fineran (2015). A combination of performing a killing assay quantified via viable count by plating and an uptake assay is even more common, as seen in Table 2.

1.2. Comparison of assays used to study *Bordetella pertussis*

Opsonophagocytic assays have been used to study varying aspects of *Bordetella pertussis* biology. Articles focusing on *B. pertussis* are marked bold in Table 2. The majority consists of uptake assays. Serology analyses comprise most articles, but several had different objective.

Several authors studied the effects of the adenylate cyclase toxin (ACT) on phagocytes. Hasan et al. focused on mechanisms of function of ACT, so the assay was adapted accordingly: uptake of zymosan was observed, without any *B. pertussis* specificity (Hasan et al., 2019). In an article describing the effects of ACT on human neutrophils, an opsonophagocytic assay was used to monitor respiratory burst. *B. pertussis* was used as target particle and was opsonized with specific anti-*B. pertussis* sera and antibodies. For optimal uptake in their assay setup, IgG proved to be the best opsonin even in complement absence (Steed et al., 1992). Inhibited phagosome-lysosome fusion and delayed respiratory burst was observed. Similar measurements were made concerning phagosome-lysosome fusion, and the conclusion was that *B. pertussis* could be capable of intracellular survival (Steed et al., 1991). Other authors also suggest that the unopsonized bacteria are able to resist intracellular killing and survive in phagocytes or epithelial cells (Lamberti et al., 2013, 2008, 2010). Concerning intracellular survival, agreement has not been reached. Another research on the same topic published by the lab of A. Weiss claims that intracellular bacteria are readily killed by human neutrophils and differences in assay outcomes are only due to attachment and uptake levels (Lenz et al., 2000). Related to this issue, targeting to receptors and role of Fc or complement receptors have been addressed for *B. pertussis*. FcγRIIa and FcγRIIIb both proved to be involved in uptake of IgG-opsonized bacteria, FcαRI in uptake of IgA opsonized targets. Dual opsonization by IgG and IgA elevated phagocytosis levels. Opsonized bacteria triggered respiratory burst in human neutrophils. Interestingly, uptake of unopsonized bacteria, which has been suggested to be CR-dependent (Mobberley-Schuman and Weiss, 2005), was low and did not induce respiratory burst. No complement-opsonized sample was included in the previously mentioned experiments (Rodriguez et al., 2001). In another opsonophagocytic assay evaluated by respiratory burst measurement, unopsonized bacteria did not induce a respiratory burst in human neutrophils (Aase et al., 2007). Mouse neutrophils also uptake IgG opsonized bacteria preferentially by FcRs (Hellwig et al., 2001). Similar trends have been observed by Lamberti and colleagues (Lamberti et al., 2008), while contrasting data have been reported by Weingart and Weiss. Here opsonization decreased attachment and phagocytosis rates by HL-60 cells (Weingart and Weiss, 2000).

Most of the articles are concerned with serology. The specificities of antibody opsonins seem to be more important than previously thought. Antibodies against pertactin were shown to be crucial for phagocytosis, phagocytosis of *Bordetella pertussis* opsonized with anti-PRN depleted sera was decreased. Such a trend was not observed after depletion of anti-PT,

anti-FHA or anti-FIM antibodies (Hellwig et al., 2003). This was contested by two different studies, where anti-PT and anti-FHA antibodies correlated with increased phagocytosis (Aase et al., 2007; Hovingh et al., 2018). Neutralizing antibodies against ACT have also proven to increase phagocytic uptake of WT strain. Not only anti-ACT antibodies provided such protection, but also antibodies against a detoxified version of the toxin (Prior et al., 2006; C. L. Weingart et al., 2000). Analysis of patient sera also indicated that opsonophagocytic activity elicited by sera wanes within 3-5 years, but still remains higher than in control sera (Hovingh et al., 2018). Curiously, several studies reported little or no increase in phagocytosis of *B. pertussis*, when opsonized by immune sera (Stefanelli et al., 2002; Christine L. Weingart et al., 2000).

As no consensus has been reached on this topic, studying the role of antibodies in opsonophagocytic immunity to *Bordetella pertussis* after immunization or infection is a main component of this thesis.

1.3.   Aims of the experimental part of the thesis

The main aim of this thesis is to optimize an opsonophagocytic assay for the measurement of activity of functional antibodies against the human pathogen *Bordetella pertussis*. The optimization of the assay consists of considering individual variables in the assay setup, such as determining the optimal cell type, particle type and evaluation method. First, sera available for testing will be characterized by antibody titer measurement and their opsonophagocytic activity will be analyzed.

2. Materials and methods

2.1. Chemicals and reagents:

Ascorbic acid: Sigma-Aldrich, USA

Bordetella pertussis agglutinogen: Testline Clinical Diagnostics, ČR

Bovine serum albumin (BSA): Sigma-Aldrich, USA

$CaCl_2$: Lach-Ner, ČR

Casein hydrolysate: Oxoid, GB

Citric acid: Lach-Ner, ČR

Dextran T500: Pharmacosmos, Denmark

Difco™ Bordet Gengou Agar Base: BD, USA

FCS: Sigma-Aldrich, USA

$FeSO_4$: Lachema, ČR

Ficoll-paque™ PREMIUM: GE healthcare, Sweden

Fluoresbrite® YG microspheres 0,75 µm: Polysciences, USA

Glycerol: Lach-Ner, ČR

H_2O_2: Lach-Ner, ČR

H_2SO_4: Lach-Ner, ČR

Heptakis(2,6-di-O-methyl)-β-cyclodextrin (cyclodextrine): Zibo Qianhui Biological Technology Co., Ltd, China

KCl: Lach-Ner, ČR

Ketamine (Calypsol®): Gedeon Richter, Hungary

KH_2PO_4: Sigma-Aldrich, USA

L-cysteine: Sigma-Aldrich, USA

L-glutathione: AppliChem, Gemany

$MgCl_2$: Sigma-Aldrich, USA

Na_2HPO_4: Lach-Ner, ČR

NaCl: Lach-Ner, ČR

NaH_2PO_4: Lach-Ner, ČR

Nicotinic acid: Sigma-Aldrich, USA

o-phenylenediamine dihydrochloride (OPD): Sigma-Aldrich, USA

Percoll: GE Healthcare, Sweden

Proline: Sigma-Aldrich, USA

RPMI: Sigma-Aldrich, USA

Sodium citrate: Lachema, ČR

Sodium glutamate: Sigma-Aldrich, USA

Tissue-culture H_2O: IMG AVČR, ČR

Trucount beads: BD, USA

tris(hydroxymethyl)aminomethane (Tris-base): Serva, Germany

tris(hydroxymethyl)aminomethane-hydrochloride (Tris-HCl): Serva, Germany

Tween® 20: Sigma-Adrich, USA

Xylazine hydrochloride: Sigma-Adrich, USA

2.2. Solutions and buffers:

Alsever solution:

0,42 % (w/v) NaCl, 0,8 % (w/v) trisodium citrate, 0,055 % (w/v) citric acid, 2,05 % (w/v) glucose; (Sigma-Aldrich, USA)

Saline 0,9 %:

0,9 % NaCl (w/v) (Baxter, USA)

Phosphate buffer saline (PBS):

138 mM NaCl, 3 mM KCl, 12 mM Na_2HPO_4, 22 mM KH_2PO_4

PBS supplemented with Tween® (PBST):

138 mM NaCl, 3 mM KCl, 12 mM Na_2HPO_4, 22 mM KH_2PO_4, 0,05 %(v/v) Tween® 20

Krebs-Ringers buffer:

Fraction A: 17,4 mM Na_2HPO_4, 3,5 mM NaH_2PO_4

Fraction B: 0,9 mM $CaCl_2$, 3,5 mM KCl, 0,9 mM $MgCl_2$, 137 mM NaCl

Citrate buffer

50 mM citric acid, 50 mM Na_2HPO_4

Bordet-Gengou (BG) agar with 15 % sheep blood:

3 % (w/v) BG agar base, 1 % (v/v) glycerol, 15 % (v/v) defibrinated sheep blood

Stainer-Scholte medium:

Fraction A: 40,36 mM Tris-HCl, 9,74 mM Tris-base, 57,28 mM sodium glutamate, 2,08 mM proline, 42,77 mM NaCl, 3,67 mM KH_2PO_4, 2,68 mM KCl, 0,49 mM $MgCl_2$, 5 % (w/v) casein hydrolysate, 1 % (w/v) cyclodextrine

Fraction B: 0,33 mM cysteine, 0,113 mM ascorbic acid, 0,033 mM nicotinic acid, 0,322 mM glutathione

Hanks' balanced salts solution (HBSS Ca,Mg+):

0,8 % (w/v) NaCl, 0,04 % (w/v) KCl, 0,006 % (w/v) KH_2PO_4, 0,0048 % Na_2HPO_4, 0,01 % (w/v) $MgSO_4 \cdot 7\ H_2O$, 0,0185 % (w/v) $CaCl_2 \cdot 2\ H_2O$, 0,01 % (w/v) $MgCl_2 \cdot 6\ H_2O$, 0,01 % (w/v) glucose, 0,035 % (w/v) $NaHCO_3$ (IMG AVČR, ČR)

Glucose:

25 % (w/v) glucose (IMG AVČR, ČR)

Hypotonic saline:

0,2 % (w/v) NaCl

Hypertonic saline:

1,6 % (w/v) NaCl

Horseradish peroxidase substrate:

(w/v) OPD, 0,03 % (v/v) H_2O_2 in citrate buffer

6 % dextran:

6 % (w/v) dextran T500 in 0,9 % saline

Anesthesia:

1 % (v/v) xylazine, 20 % (v/v) ketamine (Calypsol®)

2.3. *Bordetella pertussis* strains:

CIP 81.32, derivative of Tohama I isolate strain (Institute Pasteur, France)

CIP 81.32, derivative of Tohama I isolate strain, expressing PBBR1 plasmid, which encodes mScarlet fluorescent protein (Institute Pasteur, France)

2.4. Mouse sera:

Mouse sera were acquired from vaccination experiments performed in our laboratory. Sera were aliquoted by 50 µl. The sera were kept at -80 °C.

'Naïve'
Pooled serum from 5 female naive mice, sacrificed at 10 weeks of age

'aP vaccinated'
Pooled serum from 5 female mice vaccinated at 5 weeks of age with ¼ human dose Hexacima, sacrificed at 10 weeks of age

'aP + FIM + ACT vaccinated'
Pooled serum from 5 female mice vaccinated at 5 weeks of age with ¼ human dose Hexacima+ 3 µg/ml Fimbriae type 2/3 + ACT, sacrificed at 10 weeks of age

'wP vaccinated'
Pooled serum from 5 female mice vaccinated at 7 weeks of age with with ¼ human dose wP vaccine, sacrificed at 10 weeks of age

'Convalescent'
Pooled serum from 5 female mice infected with CIP 81.32 10^5 CFU at 5 weeks of age, sacrificed at 10 weeks of age

2.5. Human sera

Human sera were prepared as described later (Chapter 2.11.). Sera were aliquoted by 100 µl and kept at -80 °C.

'Testline'
Bordetella parapertussis-AR-Positive Control lyophil. (cat. Nr: BppP01) serum was purchased from Testline Clinical Diagnostics, ČR

Healthy donor serum
Serum was prepared from blood drawn freshly at Thomayer Hospital transfusion unit of 5 healthy human volunteers, aged 20-60, both sexes. Written informed consent was provided by donors. Samples were anonymised and labled 'Donor 1-5'.

2.6. Human blood

Human blood was drawn freshly at Thomayer Hospital transfusion unit from Donors 1 or 2. Written informed consent was provided by donors.

2.7.   Antibodies

Table 4 – Antibodies used in the thesis

Antibody	Clone	label	Host species	Target species	Manufacturer (product id)	Concentration	Final dilution
Anti-Mouse IgG	NA931V	HRP	Sheep	Mouse	GE (NAV931-1ML)	unknown	1:3000
Anti-Mouse IgG2a	polyclonal	HRP	Goat	Mouse	Abcam (ab97245)	1 mg/ml	1:50000
Anti-Mouse IgG1	polyclonal	HRP	Goat	Mouse	Invitrogen (A10551)	1 mg/ml	1:1000
Anti-Mouse Ly6G	1A8	PE-Cy7	Rat	Mouse	Biolegend (127617)	0,2 mg/ml	1:400

2.8.   Plastics:

Tubes 1,5 ml: Greiner Bio-One, Germany

Tubes 15 ml: Jet Biofil, China

Tubes 50 ml: Jet Biofil, China

Tissue culture 96-well plates, U-shape: TPP, Switzerland

Petri dish: Gama Group, ČR

Serological pipettes 25 ml: TPP, Switzerland

Serological pipettes 10 ml: Thermo Fisher Scientific, USA

Serological pipettes 5 ml: Thermo Fisher Scientific, USA

ELISA 96-well plates: Maxisorp NUNC immunoplate; Thermo Fisher Scientific, USA

Cell strainer 70 μl: Corning, USA

Vacuette K$_3$EDTA 9 ml: Greiner Bio-One, Germany

Vacutainer Sodium Heparin 6 ml: BD, USA

2.9.   Instruments software:

Centrifuge: Rotanta 460 R, Hettich, Germany

Microcentrifuge: 5424 R, Eppendorf, Germany

CO2 incubator: MCO-18 AIC, Sanyo, Japan

Spectrometer: CO8000 Cell density meter, Biochrom WPA, Great Britain

Heated shaker: PST-60 HL plus Thermo Shaker, Biosan, Latvia

Flow cytometer: LSRII, BD, USA

Vortex: Minishake 1, IKA-Works International, USA

Microplate reader: Safire[2], Tecan, Switzerland

FlowJo version 10.6.0 software

Graphpad Prism 8 software

2.10. Serum preparation

Human blood was freshly drawn and left to coagulate for 30 minutes. Sera were centrifuged 15 minutes at 1300 g. Serum was aspirated.

2.11. Heat-inactivation of serum

Aliquots of 100 µl (human) or 50 µl (mouse) were taken from each serum. The sera were heated at 56 °C for 30 minutes in a heated shaker.

2.12. Heat-killing of *Bordetella pertussis*

Non-fluorescent WT and mScarlet expressing WT *B. pertussis* were grown overnight at 37 °C, 210 RPM, to an OD_{600} of 1 in Steiner-Scholte medium. Aliquots of the culture were heated at 56 °C for 30 minutes in a heated shaker. Killing was confirmed by plating on BG agar plates, which were incubated in 37 °C, 5 % CO_2 for 5 days. Viable CFU count of both the original bacterial suspension and the heated suspension was enumerated.

2.13. Evaluation of anti-*Bordetella pertussis* IgG titers in mouse and human sera:

Whole-cell ELISA was used to evaluate the IgG antibody content of mouse and human sera. *Bordetella pertussis* was grown in Steiner-Sholte medium overnight at 37 °C 210 RPM, to OD_{600} 1. The bacterial suspension was diluted 40x in PBS. 50 µl of diluted suspension was loaded into a NUNC Maxisorp 96-well microtiter plate. The plate was incubated overnight in 37 °C without cover. 120 µl of 1 % BSA in PBS was added to each well and incubated for 1 hour in 37 °C. The wells were washed two times with PBST. The tested sera were diluted in 0,5 % BSA in PBST to target concentration. 100 µl of diluted sera were pipetted into the plate

and were incubated for 1 hour at 37 °C. Wells were emptied and washed four times with PBST. 100 µl of anti-IgG or anti-isotype antibody specific for tested species diluted in 1 % BSA in PBST was added to each well and was incubated for 50 minutes at 37 °C. Wells were washed three times in PBST. 100 µl peroxidase substrate was added into each well and incubated for 10 minutes in 37 °C protected from light. 50 µl of 2M sulfuric acid was added to each well to stop the reaction. Concurrently, blank wells were prepared by the same protocol in the same plate, only without addition of the serum (the wells contained antigens and secondary antibody). The absorbance at 492 nm was measured by Safire 2, reference wavelength used was 620 nm. For determination of the total IgG titer of mouse sera, anti-mouse IgG was used. For determination of isotype titers of mouse sera, anti-mouse IgG1 and anti-mouse IgG2a was used. For determination of the total IgG titer of human sera, anti-human IgG was used (Chapter 2.7. Antibodies, Table 3).

2.14. Antibody agglutination test:

To test the agglutination activity of anti-*Bordetella pertussis* antibodies in mouse and human sera the Agglutination reagents from Testline Clinical Diagnostics were used. First, 50 µl of 0,9 % saline was pipetted into each well. 50 µl of positive control, tested sera and negative control (0,9 % saline) were pipetted into the first column of 96-well plate with U-shaped wells. The first column of the plate was mixed. Serial 2-fold dilution was done at each row. The concentrated Bordetella pertussis-AR-agglutinogen (Testline Clinical Diagnostics, ČR) was diluted 10x in 0,9 % saline. 50 µl of the diluted agglutinogen was added into each well and mixed. The plate was incubated at 37 °C, 5 % CO_2, for 2,5 hours and then left for 18-20 hours at 20 °C. Agglutinated material diffused throughout the whole well marked a positive reaction, settled material at the bottom of a well marked a negative reaction. The agglutination titer was evaluated as the last dilution with a positive result.

2.15. Isolation of mouse neutrophils from bone marrow:

To harvest mouse neutrophils from bone marrow, mice were euthanized by cervical dislocation. Mice were sprayed with 70 % ethanol and femurs and tibias were carefully dissected. Excess connective and muscle tissue was removed. Femurs and tibias were placed in RPMI medium supplemented with 10 % FCS and kept on ice. Each bone was sterilized by 70 % ethanol and washed in sterile PBS, then the epiphyses were removed by sterile scissors

and each bone was flushed with syringe and needle with RPMI supplemented with 10 % FCS and 2 mM EDTA. Bone marrow cells are harvested into a 15 ml tube. The epiphyses of each bone were crushed by a sterile 1,5 ml tube top and strained through a 70 μm cell strainer. The bone marrow cells were pelleted by centrifuging 7 minutes at 430 g at 4 °C. The pellet was resuspended in 5 ml 0,2 % saline to lyse erythrocytes and immediately 5 ml of 1,6 % saline was added. The cells were centrifuged for 7 minutes at 430 g at 4 °C. The pellet was washed once with 5 ml of RPMI supplemented with 10 % FCS and resuspended in 2 ml of RPMI supplemented with 10 % FCS. Carefully, 3 ml of Percoll with density 1,119 g/l was overlaid by 3 ml of Ficoll with density of 1,077 g/l and then was overlaid by the bone marrow cell suspension in RPMI supplemented with 10 % FCS. The gradient was centrifuged for 30 minutes at 870 g at 20 °C. The neutrophils were aspired by pipette from the interface between the two densities and were washed twice with 5 ml of RPMI supplemented with 10 % FCS. The cells were resuspended in RPMI supplemented with 10 % FCS and counted in a Bürker chamber. Neutrophils were labeled by anti-Ly6G antibody, viability was determined by Hoechst 33258 staining and measured by flow cytometry.

2.16. Isolation of mouse leucocytes from peripheral blood by erythrocyte lysis:

To collect blood, mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (8 mg/kg) in saline. Blood was drawn from orbital plexa directly into heparinized blood collection tubes. The blood was then transferred to a new tube. For lysis of erythrocytes, 20 ml of hypotonic saline was added to each 5 ml of blood and gently mixed by inversion of the tube. After 20 seconds 20 ml of hypertonic saline was added and gently mixed by inversion of the tube. The tubes were centrifuged for 6 minutes at 340 g at 20 °C. The supernatant was removed, and the pellet resuspended in 0,5 ml of 0,9 % saline. The lysis steps were repeated 3-5 times, until erythrocyte lysis was accomplished. The cells were washed twice and resuspended in HBSS +Ca,Mg supplemented with 1 % BSA. Neutrophils were labeled by anti-Ly6G antibody, the amount and percentage of granulocytes was counted by flow cytometry. Viability was determined by Hoechst 33258 staining.

2.17. Isolation of human neutrophils on plasma-Percoll gradient:

40 ml of blood was aspired into Vacuette tubes coated with anticoagulant: EDTA or sodium heparin. 4 ml of Alsever solution was added. The blood was centrifuged for 20 minutes at 340 g at 20 °C without brakes to avoid disturbing the gradient. The platelet rich plasma supernatant was aspired and centrifuged for 30 minutes at 2180 g at 20 °C, generating platelet poor plasma. 6 ml of 6 % dextran was added to the pelleted cells, the tube was filled up to 50 ml with 0,9 % saline and left for 1 hour to sediment. After sedimentation, the supernatant was collected and centrifuged for 6 minutes at 340 g at 20 °C. The pellet was resuspended in 2 ml platelet poor plasma. A plasma-Percoll gradient was prepared. Two densities of Percoll were prepared:

"a" – 840 µl of 90 % Percoll and 1160 µl of platelet poor plasma

"b"- 1020 µl of 90 % Percoll and 980 µl of platelet poor plasma

The resuspended cells were underlaid with layer "a" and subsequently with layer "b". The gradient was centrifuged for 10 minutes at 290 g and 20 °C without brakes. Neutrophils were harvested from the interface of the two densities, the second puffy layer from the top of the gradient. The neutrophils were centrifuged for 6 minutes at 340 g at 20 °C. The pellet was resuspended in 5 ml of hypotonic saline to lyse erythrocytes and immediately 5 ml of hypertonic saline was added. The neutrophils were washed twice in HBSS and counted in a Bürker chamber. Viability was determined by Hoechst 33258 staining on flow cytometer.

2.18. Isolation of human leucocytes by dextran sedimentation:

40 ml of blood was drawn into tubes coated with anticoagulant: EDTA or heparin. The blood was mixed with 6 ml of 6 % dextran and 4 ml of 0,9 % saline. The blood was left to sediment for 1 hour, or untill the interface between pelleted erythrocytes and supernatant was clear. The supernatant was then aspired and centrifuged for 6 minutes at 340 g at 20 °C. The pellet was resuspended in 5 ml hypotonic saline to lyse contaminating erythrocytes and immediately after 5 ml of 1,6 % saline was added. The cells were washed twice and resuspended in HBSS +Ca,Mg supplemented with 1 % BSA. The amount and percentage of granulocytes and monocytes was assessed by flow cytometry, using Trucount beads (BD, USA). Viability was determined by Hoechst 33258 staining.

2.19. Isolation of human leucocytes by erythrocyte lysis:

40 ml of blood was drawn into tubes coated with anticoagulant: EDTA or heparin. The blood was aliquoted by 5 ml into 50 ml centrifugation tubes. For lysis of erythrocytes, 20 ml of hypotonic saline was added to each 5 ml of blood and gently mixed by inversion of the tube. After 20 seconds 20 ml of 1,6 % saline was added and gently mixed by inversion of the tube. The tubes were centrifuged for 6 minutes at 340 g at 20°C. The supernatant was removed, and the pellet resuspended in 0,5 ml of 0,9 % saline. The lysis steps were repeated 3-5 times, until the pellet was clear of erythrocytes. The cells were washed twice and resuspended in HBSS +Ca,Mg supplemented with 1 % BSA. The amount and percentage of granulocytes and monocytes was counted by flow cytometry, using Trucount beads (BD, USA. Viability was determined by Hoechst 33258 staining.

2.20. Bead OPA:

Fluoresbrite beads (Polysciences Inc., USA) were diluted to 10^8 particles/ml in Krebs-Ringers buffer. For opsonization, tested serum was diluted to 50 % concentration with Krebs-Ringers buffer. 10^6 particles in 10 µl were added to the diluted sera and incubated for 30 minutes at 37 °C in a heated shaker. For opsonophagocytic uptake, 100 µl of cells (isolated neutrophils/granulocytes or leucocytes) in Krebs-Ringers buffer (roughly 10^5 cells) were mixed with 10 µl of opsonized beads (5 % serum concentration) in a 96-well plate. The multiplicity of infection was 1:1. The cells were incubated with the beads for 30 minutes in 37 °C in a heated shaker. After incubation, 100 µl of ice-cold PBS was added to stop phagocytosis. The cells were washed twice with and resuspended in 100 µl of ice-cold PBS. The plate with samples was kept on ice until analyzed on flow cytometer. Two technical replicates were prepared for each sample. The parameter studied were the percent of phagocytic cells, defined as cells containing at least one particle.

2.21. *Bordetella pertussis* OPA:

Bordetella pertussis expressing mScarlet fluorescent protein was grown overnight in Steiner-Scholte medium at 37 °C, 210 RPM. After reaching OD_{600} 1, the bacteria were heat-killed (Chapter 2.12.). 5 µl of tested sera were aliquoted to a 96-well plate and 40 µl of HBSS +Ca,Mg supplemented with 1 % BSA was added to each well. 5 µl of bacteria were added to the diluted sera, final serum concentration was 10 %. The serum with bacteria was incubated for 30 minutes at 37 °C in a heated shaker. For opsonophagocytic uptake, 50 µl of cells (containing

$3 \cdot 10^5$ granulocytes) was added. Cells were incubated with the bacteria for 30 minutes at 37 °C in a heated shaker. After incubation, 100 µl of ice-cold PBS was added to stop phagocytosis. The cells were washed twice with and resuspended in 100 µl of ice-cold PBS. Samples were kept on ice until analyzed by flow cytometry. Two technical replicates were prepared for each sample. Mean fluorescence intensity of the ingested bacteria was measured.

2.22. *Bordetella pertussis* opsonophagocytic killing assay:

Bordetella pertussis was grown overnight in Steiner-Scholte medium at 37 °C, 210 RPM, to OD_{600} 1. For opsonization, 2,5 µl of tested sera were aliquoted to a 96-well plate and 40 µl of HBSS +Ca,Mg supplemented with 1 % BSA was added to each well. 5 µl of bacteria were added to the diluted sera, final serum concentration was 5 %. The serum with bacteria was incubated for 30 minutes at 37 °C in a heated shaker. For opsonophagocytic uptake, 50 µl of cells (containing $3 \cdot 10^5$ granulocytes) was added. Cells were incubated with the bacteria for 60 minutes at 37 °C in a heated shaker. After incubation, the plate was placed on ice and each sample was serially diluted. Three dilutions were plated on BG agar plates. CFUs were counted after 5-8 days of incubation in 37 °C and 5 % CO_2.

2.23. Statistics

Data from the *BP* OPA assay were analyzed by two-way ANOVA followed by Dunett's test for comparison of subgroups to the 'no serum' control. Each sample was compared with the 'no serum' control. *, ** and *** represent P values of 0.05, 0.01 and 0.001, respectively.

3. Results

3.1. Determination of antibody titer in mouse serum by ELISA method

To characterize the available mouse sera, total IgG titers of the sera were determined by an enzyme-linked immunosorbent assay (ELISA). The titers were obtained by serial dilution of the serum samples and performing indirect ELISA with whole-cell *Bordetella pertussis* as antigen. Anti-mouse IgG secondary antibody (Chapter 2.7. Antibodies, Table 3) was used for detection. The obtained data were fitted with a 4-point sigmoidal curve (Figure 2). Titers were determined as values corresponding to 2 × blank average (Winter and Barenkamp, 2016). The values were interpolated from the fitted curve and rounded off.
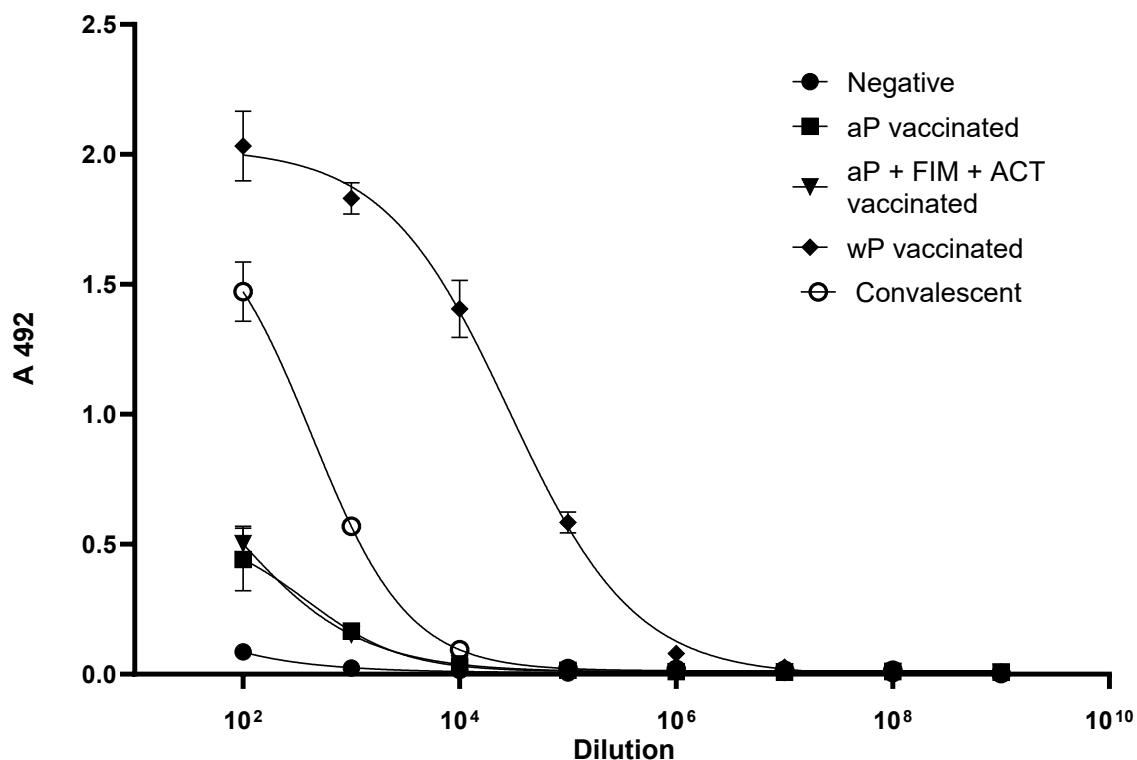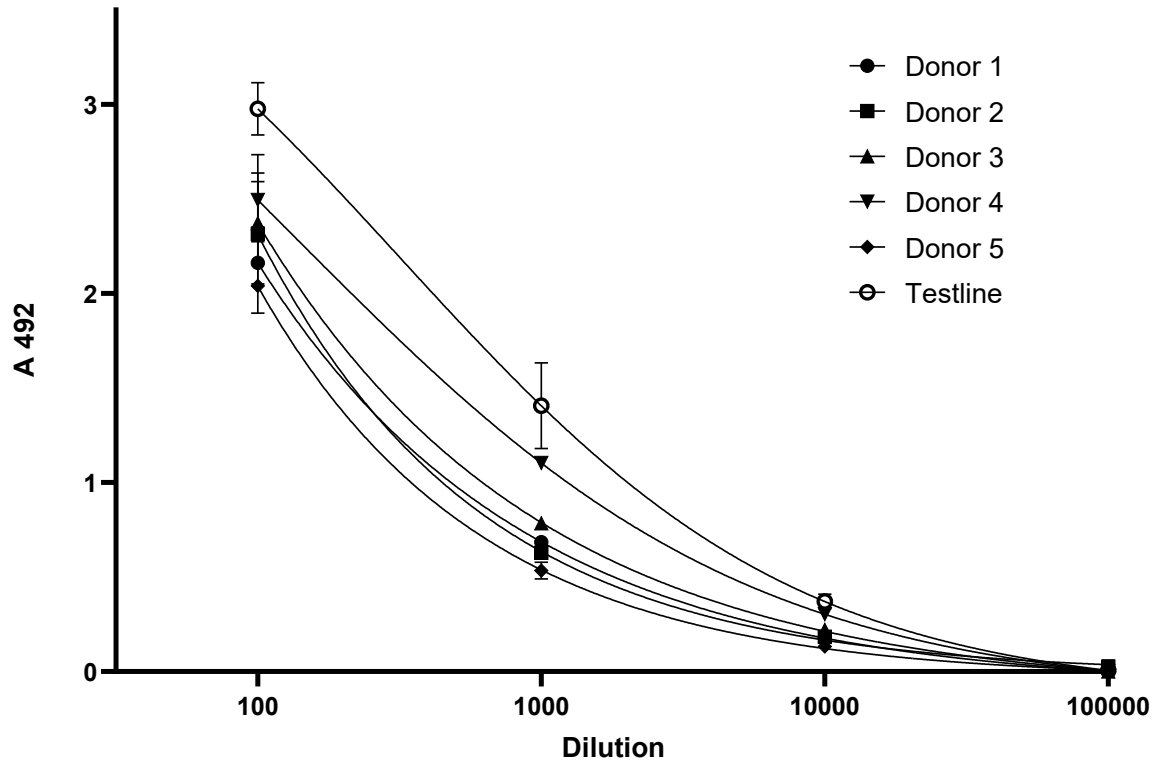

Figure 2 – Anti-*Bordetella pertussis* total IgG of mouse sera – Serum samples were serially diluted and indirect ELISA was performed with whole-cell *Bordetella pertussis* as antigen. The data was fitted with 4-point sigmoidal curves.

The curves obtained by the interpolation illustrate the differences between tested sera and correspond to the titers, which are listed in Table 5. The naïve serum had a titer of 1,000, while the highest titer was 9,762,000 for the wP vaccinated serum.

Table 5 – Total IgG titers of mouse sera

Tested serum	Titer obtained
Naive	1,000
aP vaccinated	23,000
aP + FIM + ACT vaccinated	36,000
wP vaccinated	9,762,000
Convalescent	126,000

3.2. Determination of antibody titer of IgG1 or IgG2a isotypes in mouse serum by ELISA method

Aside from total IgG titers, the IgG2a and IgG1 isotype titers of the sera were determined. These titers are relevant for the OPA and OPK assays. The data were fitted with a sigmoidal curve and titers were determined as described above.
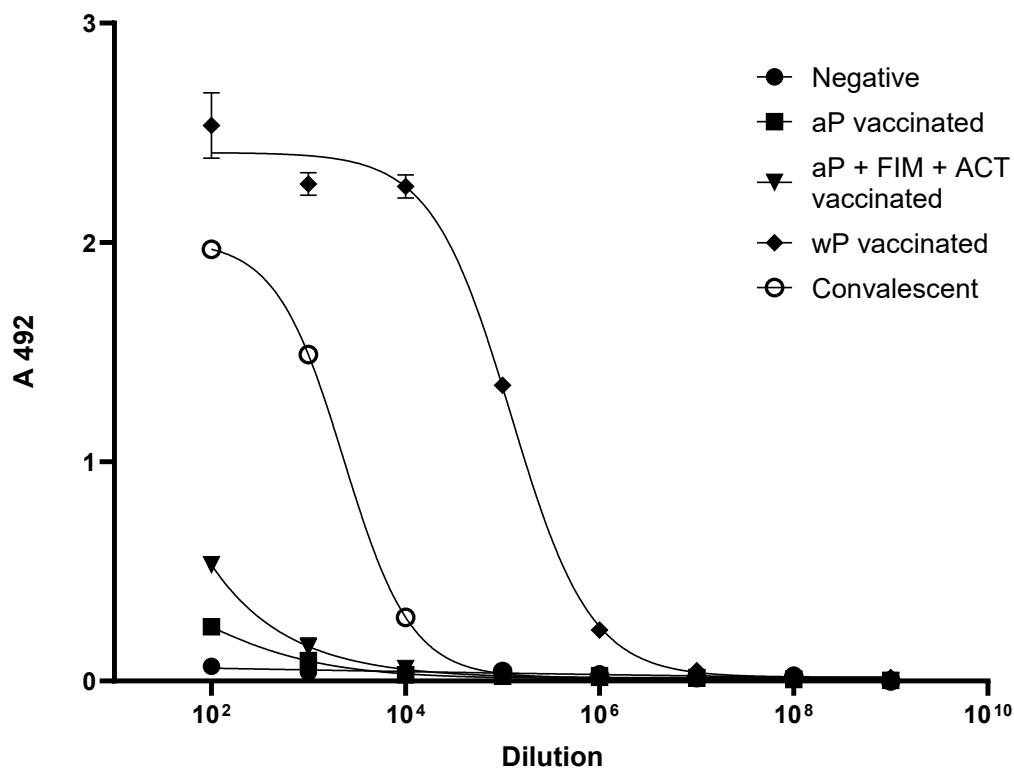
Figure 3 – Anti-*Bordetella pertussis* IgG2a of mouse sera – Serum samples were serially diluted and indirect ELISA was performed with whole-cell *Bordetella pertussis* as antigen. The secondary antibody was specific to IgG2a isotype. The data was fitted with 4-point sigmoidal curves.
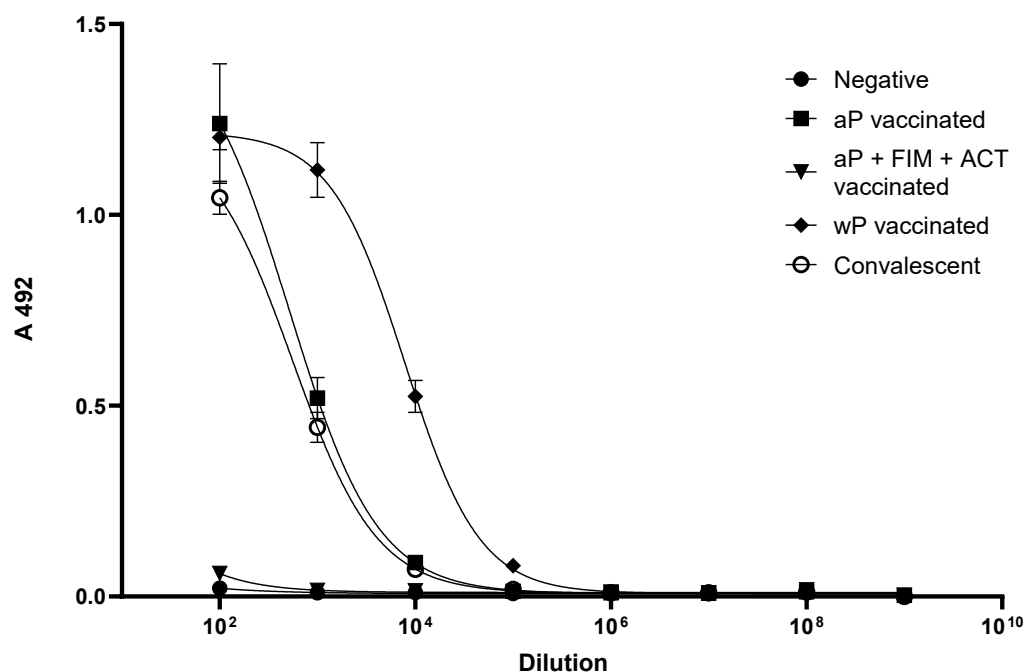
Figure 4 – Anti-*Bordetella pertussis* IgG1 of mouse sera – Serum samples were serially diluted and indirect ELISA was performed with whole-cell *Bordetella pertussis* as antigen. The secondary antibody was specific to IgG1 isotype. The data was fitted with 4-point sigmoidal curves.

As shown in Table 6, the IgG2a isotype titer obtained was lower as compared to the IgG1 isotype titer of the aP vaccinated mouse serum. In the other sera, the values of the IgG2a titers were larger than those of the IgG1 titers (Table 6).

Table 6 – IgG2a and IgG1 isotype titers of mouse sera

Tested serum	IgG2a titer	IgG1 titer
Naïve	100	100
aP vaccinated	3,000	22,000
aP + FIM + ACT vaccinated	8,000	100
wP vaccinated	5,445,000	157,000
Convalescent	49,000	17,000

3.3. Determination of antibody titer in human serum by ELISA method

Antibody titer was determined for the available human serum samples and the commercially available positive control serum from Testline Clinical Diagnostics (Testline serum). The titer values were obtained in the same way as for the mouse sera.

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Figure 5 – Anti-*Bordetella pertussis* total IgG of human sera – Serum samples were serially diluted and indirect ELISA was performed with whole-cell *Bordetella pertussis* as antigen. The data was fitted with 4-point sigmoidal curves.

As shown in Figure 5, the curves obtained by fitting the data obtained from the human serum samples were flatter and less markedly sigmoidal, than the curves obtained from the mouse sera. The titer values are listed in Table 7.

Table 7 - Total IgG titers of human sera

Tested serum	Titer obtained
Testline positive control serum	115,000
Donor 1	22,000
Donor 2	24,000
Donor 3	44,000
Donor 4	93,000
Donor 5	9,000

The values ranged from 9,000 to 93,000 for human donors, while the Testline serum had a titer of 115,000. Sera from Donors 1-3 were further used in OPA and OPK assays.

3.4. Determining the effect of heat-inactivation on antibody titer by ELISA method

To test the effect of complement in serum, aliquots of each serum were heat-inactivated (Chapter 2.11.). To analyze the effect of heat-inactivation on serum titers, the naïve, wP vaccinated, convalescent mouse sera and the Testline human serum were heat-inactivated and analyzed by ELISA as above. The antibody titers remained unchanged after heat-inactivation (Figure 6).
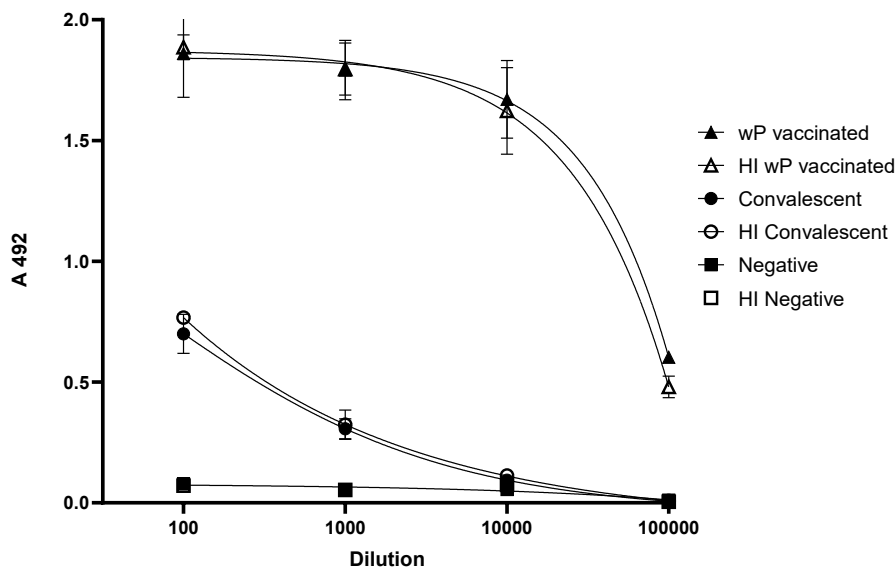
Figure 6 – Anti-*Bordetella pertussis* IgG of untreated and heat inactivated sera, **mouse serum samples** – indirect ELISA was performed with untreated and heat-inactivated aliquots of sera. Serial dilutions of each aliquot were analyzed and 4-point sigmoidal curves were fitted to the data.
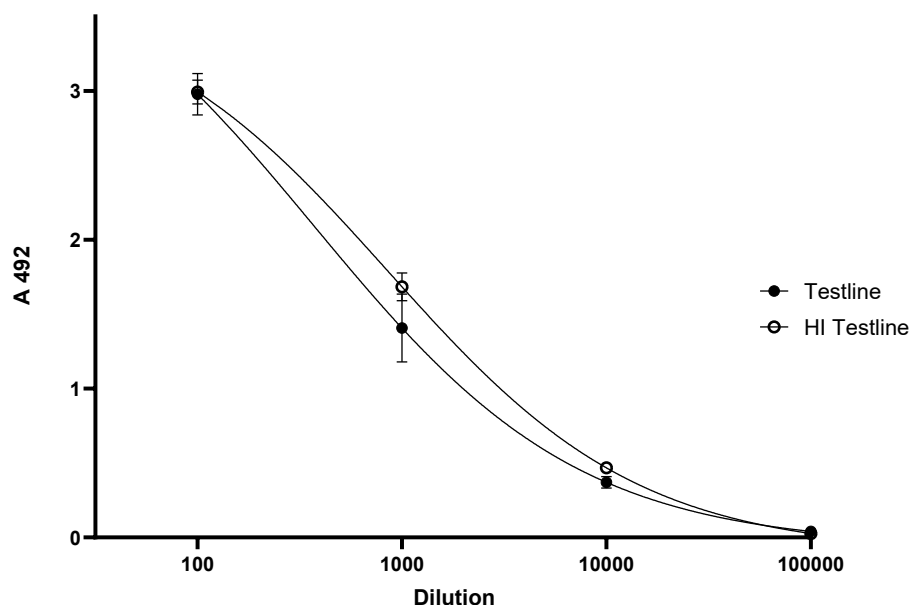
Figure 7 – Anti-*Bordetella pertussis* IgG of untreated and heat-inactivated sera, Human Testline sample – indirect ELISA was performed with untreated and heat-inactivated aliquot of the serum. Serial dilutions of each aliquot were analyzed and 4-point sigmoidal curves were fitted to the data.

3.5.   Agglutination titer determination in mouse and human sera

Agglutination titer is used in clinical diagnostics of *B. pertussis* (Zee et al., 2015) and plays an important role in the serologic analysis of sera (Hellwig et al., 2003). To test the agglutinating activity of the sera, reagents from Testline Clinical Diagnostics were used. In U-shaped wells, sera were serially diluted and incubated with agglutinogen, which is composed of inactivated *Bordetella pertussis* cells. Agglutination of the material was observed as cloudiness of the liquid. In negative samples, the agglutinogen accumulated at the bottom of the wells. Titers were determined as the last dilution to agglutinate with the bacterial cells (Tables 8 and 9).

Table 8 – Agglutinating titers of mouse sera

Tested serum	Titer obtained
Naïve	64
aP vaccinated	64
aP + FIM + ACT vaccinated	256
wP vaccinated	1,024
Convalescent	64

Table 9 – Agglutinating titers of human sera

Tested serum	Titer obtained
Testline positive control serum	1,024
Donor 1	32
Donor 2	16
Donor 3	256
Donor 4	256
Donor 5	128

The agglutinating titers of aP vaccinated and convalescent sera were of the same value as naïve serum. The wP vaccinated serum has an agglutinating titer of 1,024, the same value as the human positive control serum from Testline. human donor sera had agglutinating titers between 16 and 256.

3.6. OPK assays performed with mouse neutrophils

Swamydas et al. (2015) reported the most efficient method of neutrophil isolation. Their protocol was followed to obtain mouse neutrophils. Briefly, bone marrow was flushed from femurs and tibias of mice and the cell suspension was layered on a discontinuous gradient. Two distinct bands of cells were obtained, the upper containing mononuclear cells and the lower containing granulocytes, mainly neutrophils (Figure 8). The whole isolation procedure took about 5-6 hours before purified cells were prepared and yielded 10^6 cells per mouse. Flow cytometry analysis revealed that the cells were highly homogenous and viable (Figure 9). The percentages of neutrophils ranged from 80-95 % of single cells, with more than 95 % viability, as determined by Hoechst 33258 staining. However, the procedure was successful only in about 50 % experiments, even when the isolation technique was followed according to the established protocol. For such a low reliability, we switched to other isolation techniques.
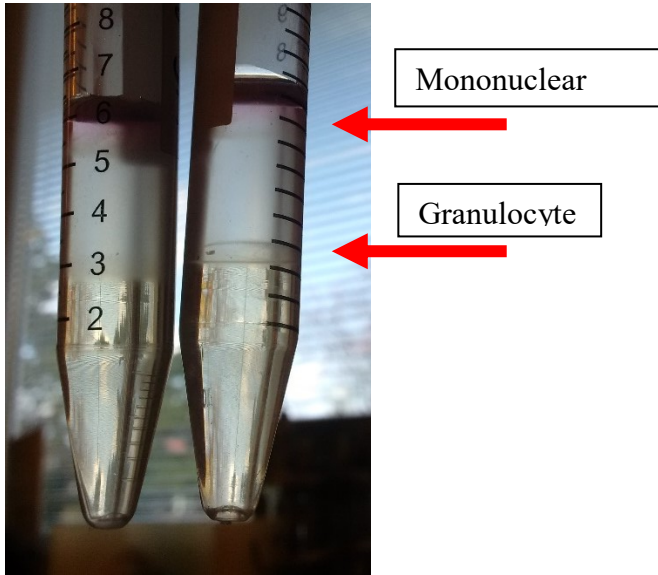
Figure 8 – Gradient with bone marrow cells after centrifugation – Mouse bone marrow was layered over a discontinuous gradient and centrifuged to separate mononuclear cells from granulocytes. The mononuclear layer is very faint at the top of the gradient, the granulocyte layer is on the interface of the densities around the 3 ml mark.
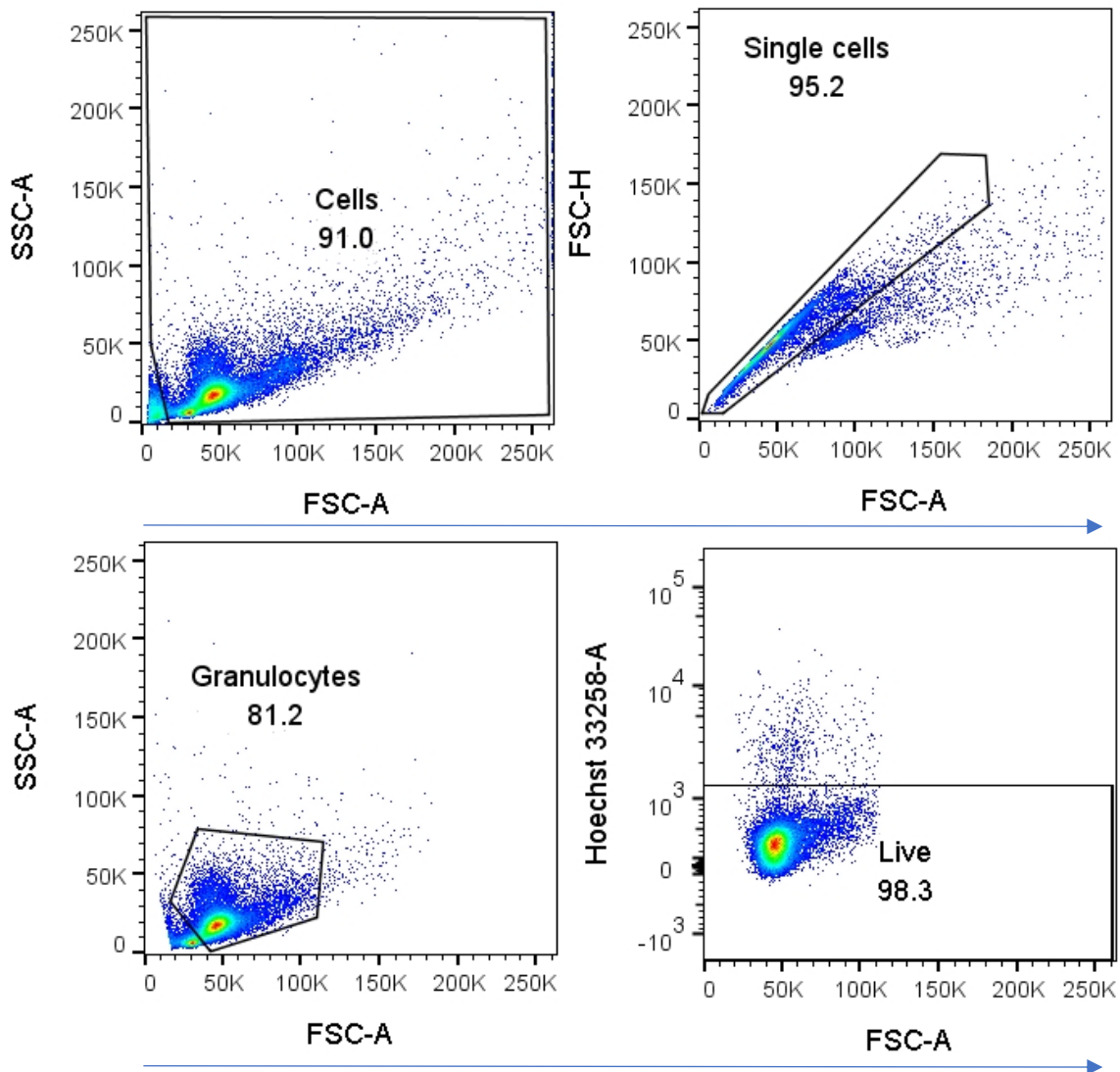
Figure 9 – Mouse neutrophils isolated from bone-marrow, purity and viability control. The cells were isolated from bone marrow by density gradient centrifugation and their purity and viability was determined by flow cytometry. The granulocyte population, containing mainly neutrophils (Swamydas et al., 2015), was counted. The viability was determined by Hoechst 33258 exclusion.

A preliminary OPK was carried out, with limited number of samples. Only sera from convalescent mice as positive control and naïve mice as negative control were used. Two dilutions of sera were tested, 10 % concentration and 2 % concentration. The sera were not heat-inactivated, and no other relevant controls were used. The MOI of 1:10 was chosen. Opsonized bacteria were incubated with neutrophils. After incubation, the suspension was serially diluted and plated onto BG agar. CFU were counted after 5 days.
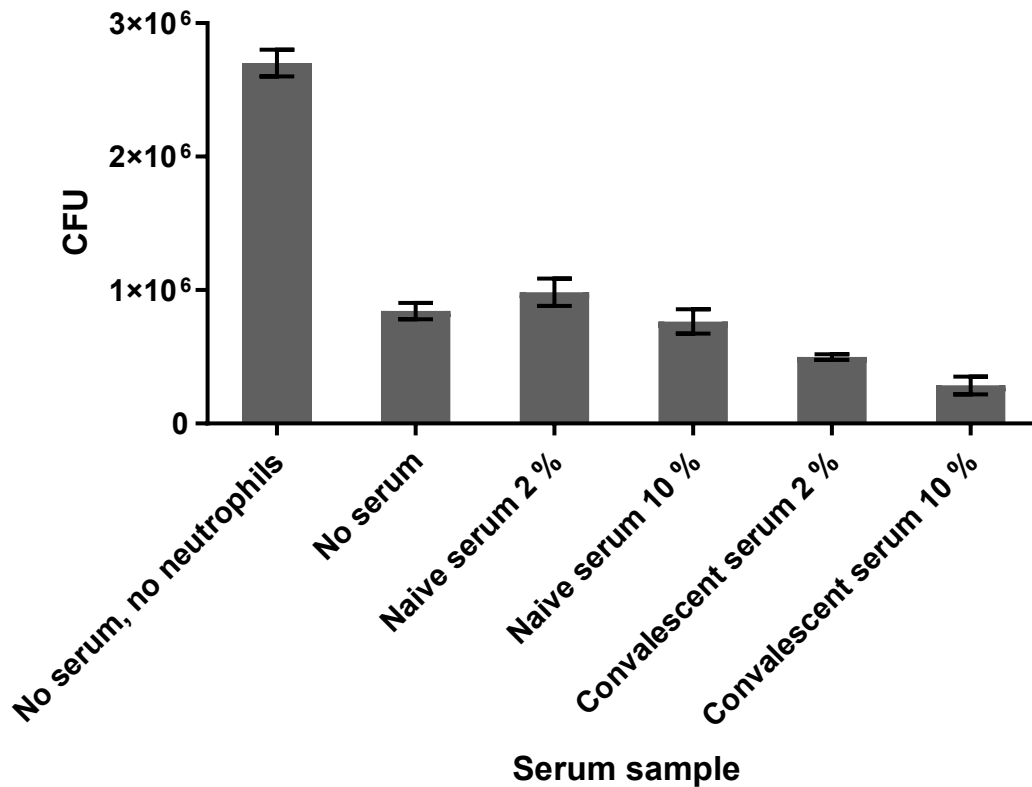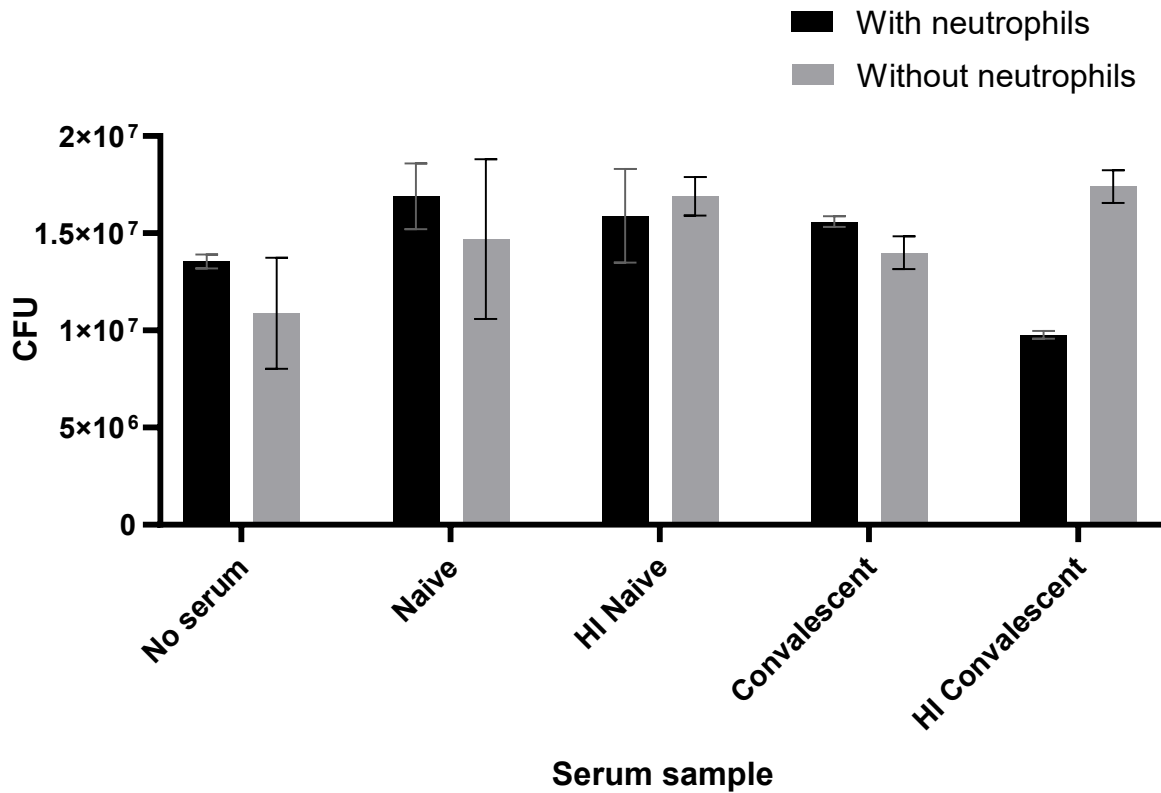
Figure 10 – OPK assay with isolated mouse neutrophils – The neutrophils were isolated by gradient centrifugation and incubated with serum samples at two dilutions. The resulting suspension was then plated on BG agar and CFU were counted after 5 days.

As shown in Figure 10, there was a marked decrease in CFU in the presence of neutrophils. Naive serum did not lower the CFU, but both concentrations of convalescent serum did. The 10 % concentration lowered the CFU to only $3 \cdot 10^5$. There was clear effect of serum, but effect of complement could not be assessed.

The OPK was then performed with the relevant controls. Untreated and heat-inactivated aliquots of sera were used and controls without neutrophils were included. The MOI was kept at 1:10 and serum concentration was 2%. As shown in Figure 11, the results differed from the preliminary experiment. No difference was observed with or without neutrophils in the sample where no serum was used. The only serum sample where addition of neutrophils lowered CFU compared to control was heat-inactivated convalescent serum. Since this difference was observable only in the heat-inactivated sample but not in the untreated sample, the data contrasts the preliminary measurements. Due to large discrepancies in the measurements and difficulties in neutrophil isolation, the work with the bone-marrow derived mouse neutrophils was discontinued.

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Figure 11 – OPK assay with isolated mouse neutrophils - The neutrophils were isolated by gradient centrifugation and incubated with untreated and heat-inactivated serum samples. Controls without neutrophils were included. The resulting suspension was then plated on BG agar and CFU were counted after 5 days.

3.7. OPK assays performed with human phagocytes

Human cells were also tested for use in the killing assays. Two methods of obtaining human neutrophils were tested in order to determine suitability for the assay. One method provides pure neutrophils, the other provides leukocytes. Both methods require fresh peripheral blood drawn into anticoagulant coated tubes.

The isolation technique was adapted from Cerny et al. (2017), only heparin was used for this assay instead of EDTA. The drawn blood was separated into cells and plasma. Erythrocytes were sedimented by addition of dextran, leukocytes were layered onto a discontinuous plasma-Percoll gradient and centrifuged. Two bands of cells were formed, the higher containing mononuclear cells and the lower containing granulocytes. Contaminating erythrocytes pelleted at the bottom of the tube. The separated cells are depicted in Figure 12.
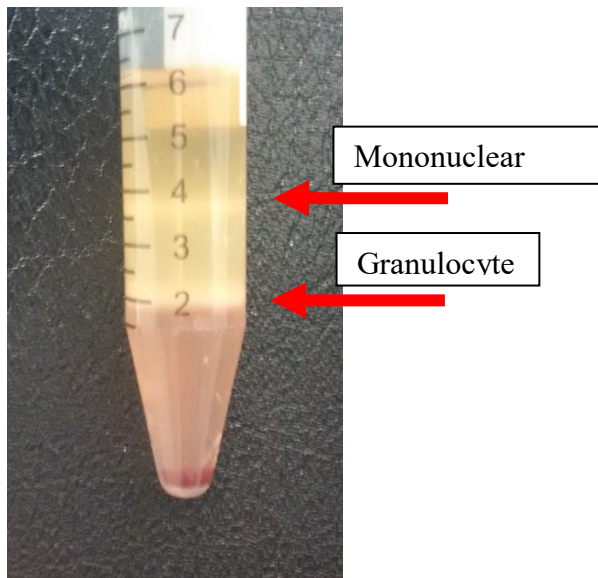
Figure 12 – Separation of human cells on plasma-Percoll gradient – Human leukocytes obtained by dextran sedimentation of peripheral blood were layered over the gradient and centrifuged. The mononuclear layer is higher, the granulocyte layer is around the 2 ml mark. Contaminating erythrocytes pelleted at the tube bottom.

Neutrophils isolated on the plasma-Percoll gradient were highly homogenous and viable. The percentage of neutrophils ranged between 90-98 %, with the viability of about 95 %. The purity and viability of neutrophils did not differ between the donors. The time required for isolation of neutrophils, measured from the drawing of blood to the last washing step, was about 4 hours. The yield of cells from 40 ml of blood was about $4 \cdot 10^7$ cells.
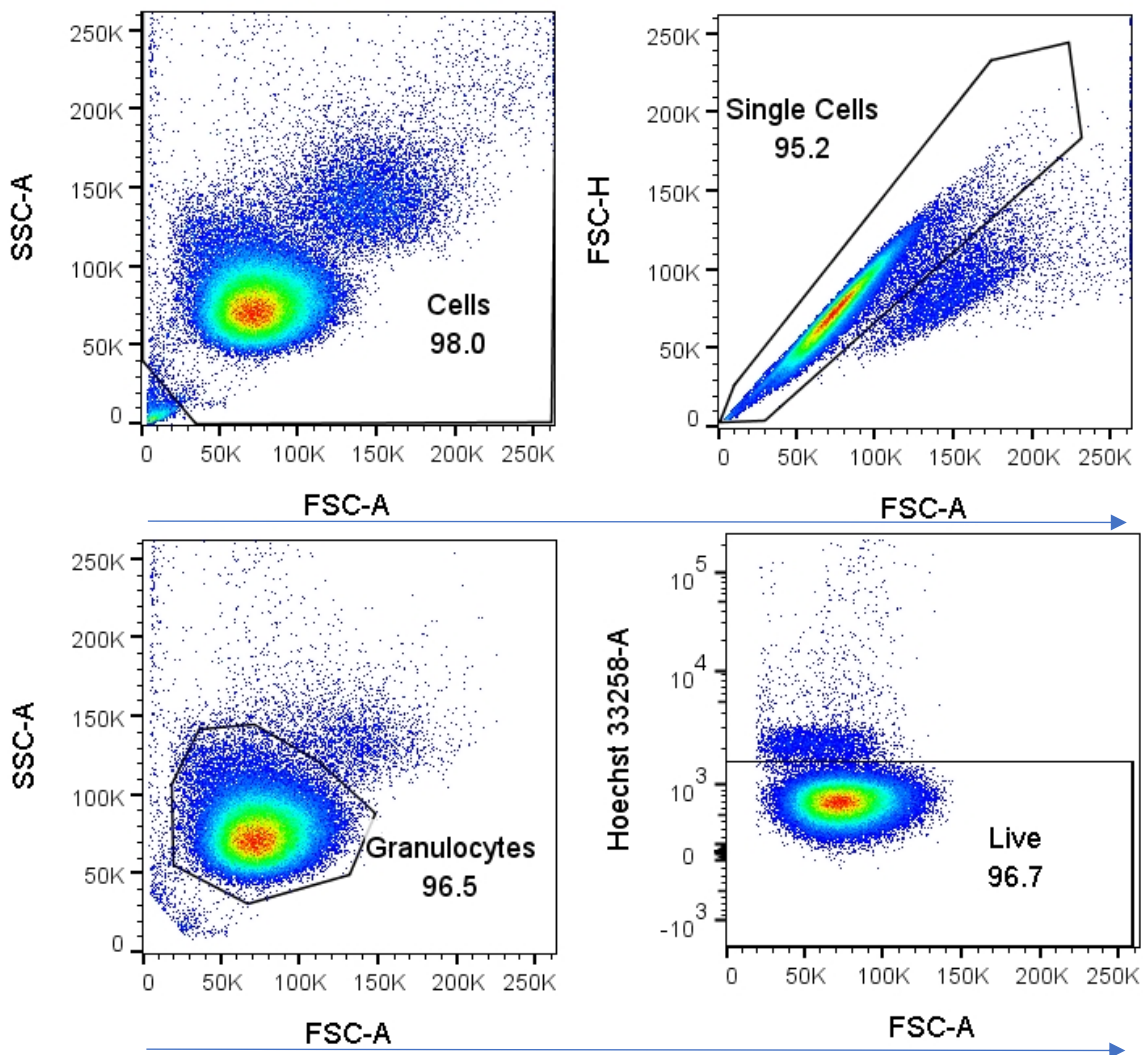
Figure 13 – Human neutrophils isolated from peripheral blood by plasma-Percoll gradient centrifugation purity and viability control. The cells were first sedimented by addition of dextran, the obtained leukocytes were then separated by density gradient centrifugation and their purity and viability was determined by flow cytometry. The granulocyte population was counted. The viability was determined by Hoechst 33258 exclusion.

To obtain erythrocyte-free leukocytes, 3-5 lysing steps were used. Lysis was performed by resuspension of whole blood in hypotonic saline, and subsequent addition of hypertonic saline. The time needed to obtain leukocytes, counted from blood draw to last cell wash, was 2-3 hours. The cell suspension contained all leukocytes. The cell count of the sum of monocyte and granulocyte populations was determined by flow cytometry. The populations differed between donors. Cells from Donor 1 contained 50-60 % granulocytes and 3 % monocytes (Figure 14). Cells from Donor 2 consisted of about 70 % granulocytes and 6-7 % monocytes (Figure 15). Cells from both donors were viable (more than 95 %), as determined by Hoechst 33258 staining. This was confirmed by three independent measurements from each donor, performed on different days.
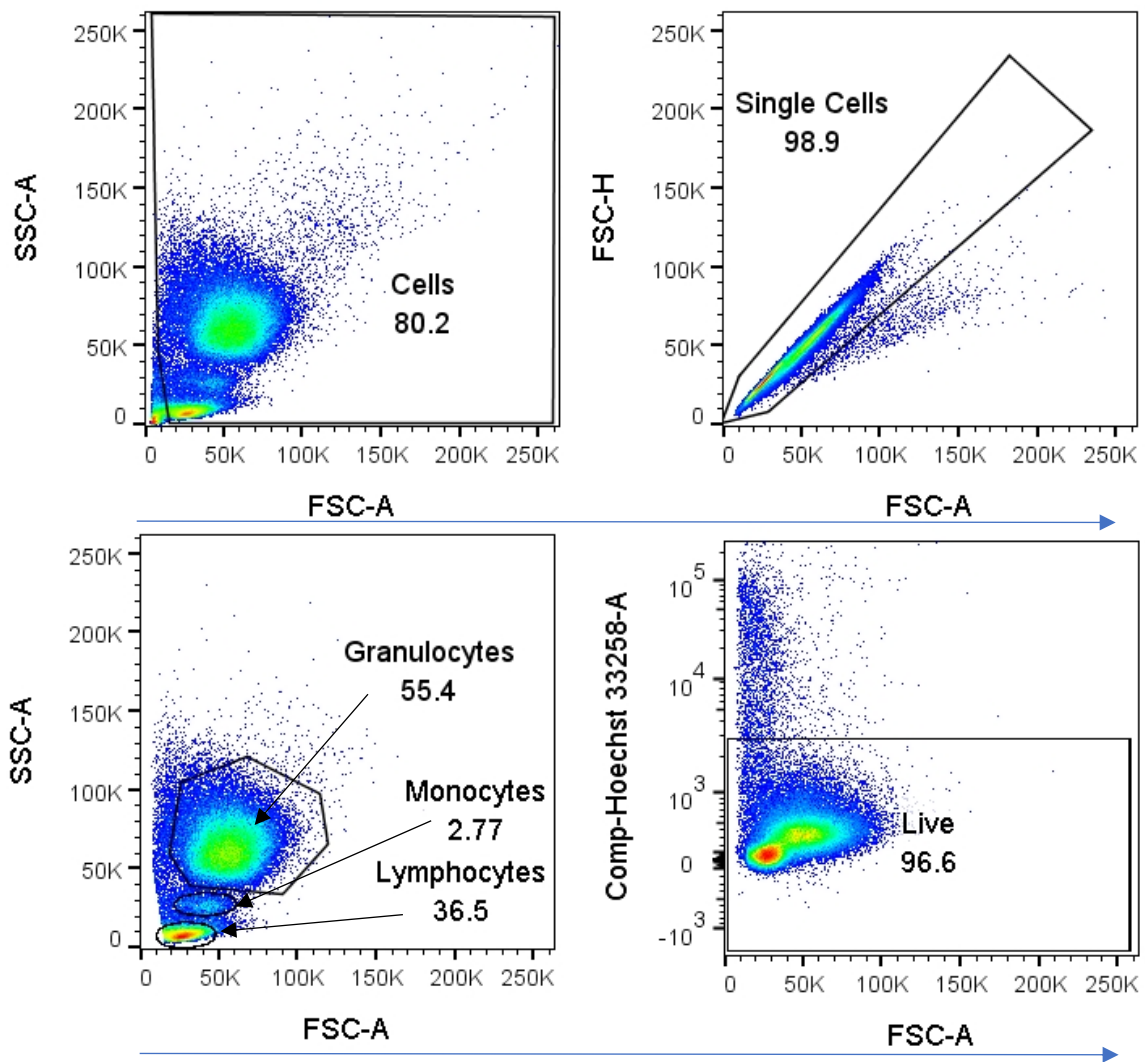
Figure 14 – Human leukocytes obtained by erythrocyte lysis of peripheral blood, Donor 1 – Erythrocytes from peripheral blood was lysed by hypotonic lysis and leukocytes were harvested. The cell population percentages were measured by flow cytometry and viability was determined by Hoechst 33258 exclusion.
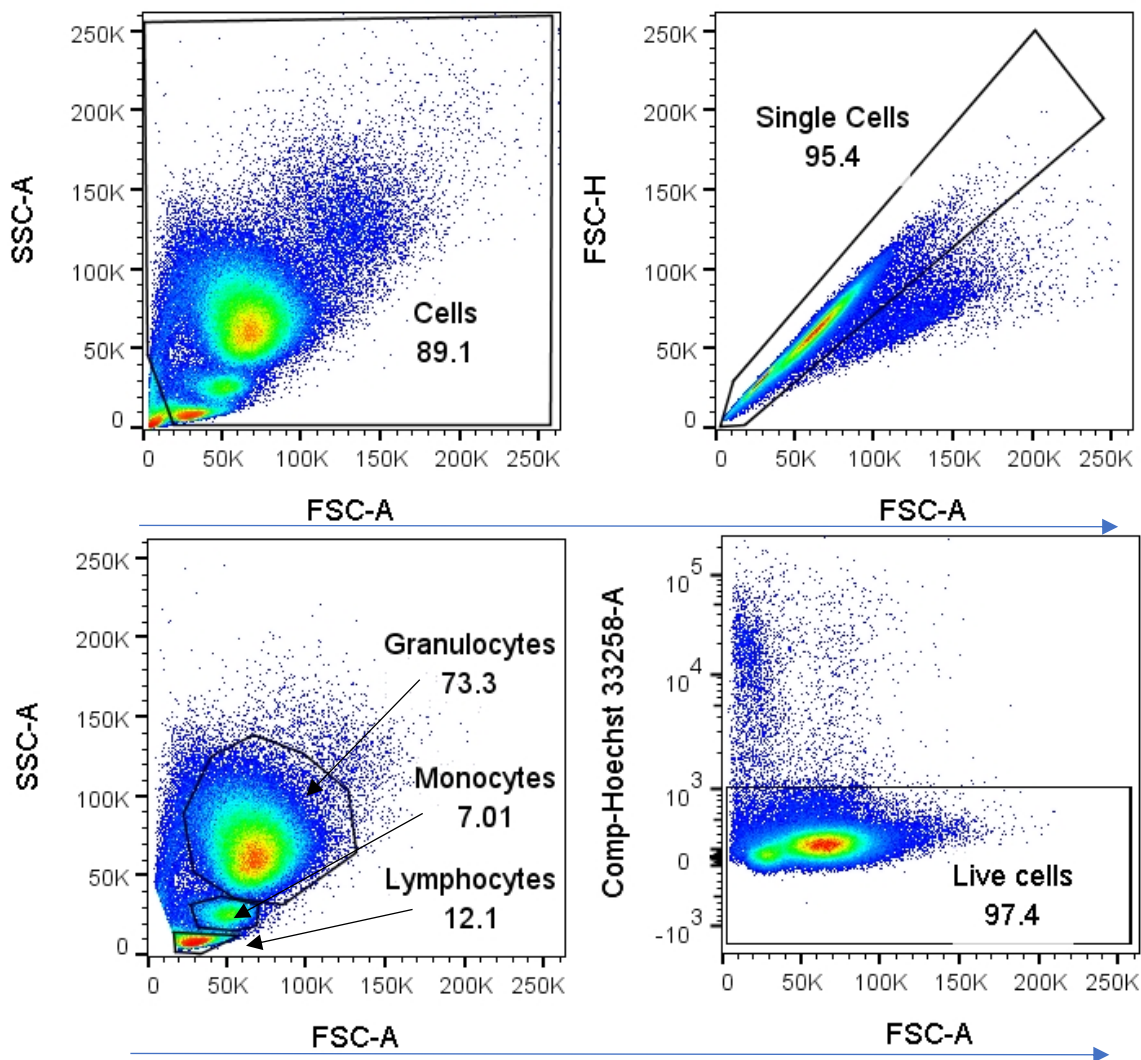
Figure 15 – Human leukocytes obtained by erythrocyte lysis of peripheral blood, Donor 2 – Erythrocytes from peripheral blood was lysed by hypotonic lysis and leukocytes were harvested. The cell population percentages were measured by flow cytometry and viability was determined by Hoechst 33258 exclusion.

Cells obtained by both methods were used for the killing assays. The OPK with plasma-Percoll isolated cells was performed twice, blood was obtained from Donor 1 for first experiment and from Donor 2 for the repeated experiment. Three serum samples were used: convalescent and naive mouse serum, as well as positive human serum purchased from Testline Clinical Diagnostics (Testline serum). A heat-inactivated aliquot of each serum was also tested. 10 % concentration of sera was used for opsonization and was further diluted to 5 % by addition of neutrophils. The MOI was set at 1:5. Controls without neutrophils were included.
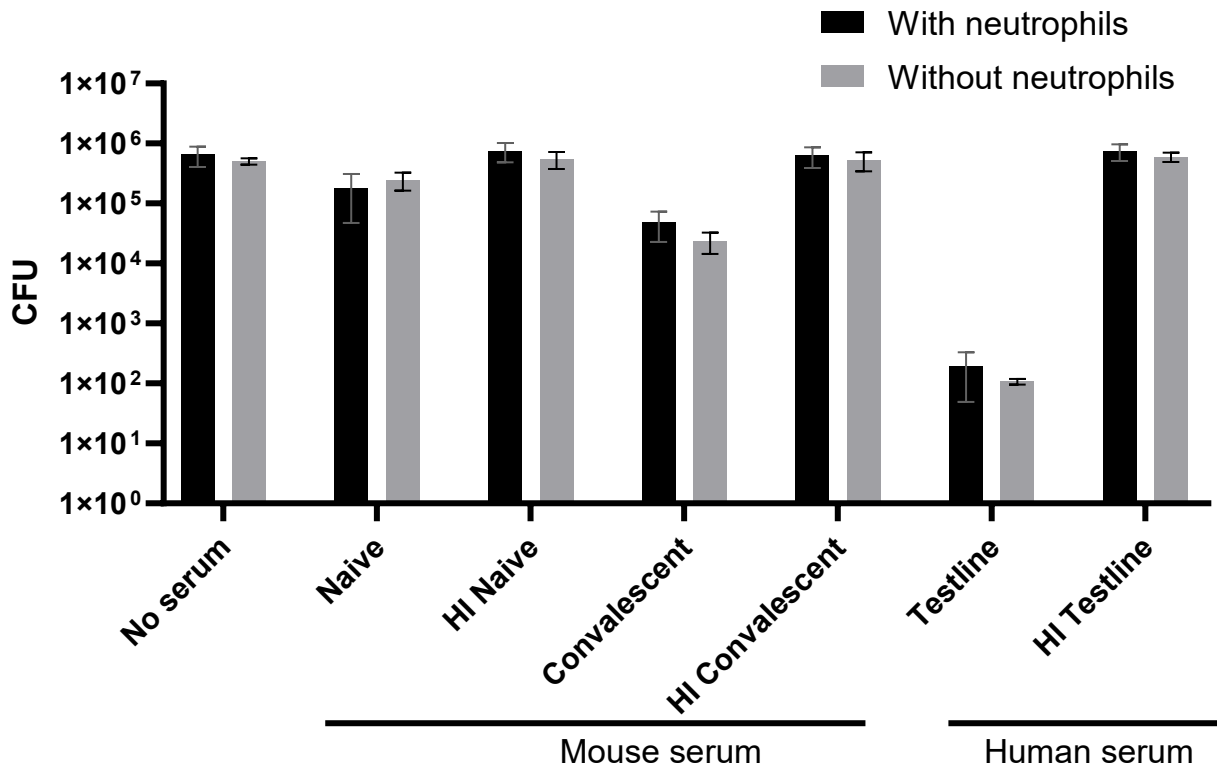
Figure 16 – OPK with isolated human neutrophils – Human neutrophils were obtained from peripheral blood by dextran sedimentation and subsequent gradient centrifugation on a plasma-Percoll gradient. Neutrophils were incubated with Bordetella pertussis cells at MOI 1:10 and indicated sera at 10 % concentration. The resulting suspension was plated on BG agar and CFU were counted after 5 days. Logarithmic scale was used on the y axis.

As shown in Figure 16, the untreated sera reduced the CFU count. The heat inactivated sera did not affect CFU count compared to control. The control without serum and neutrophils resulted in $5 \cdot 10^5$ CFU, addition of neutrophils had no effect. The Testline serum reduced the CFU to 150, and to 106 in the control without neutrophils, showing more than 99 % CFU reduction. Convalescent mouse serum reduced the CFU to $5 \cdot 10^4$, that is by 90 %. The naive serum reduced the CFU count by 50 %, with least effect. All killing was dependent on the presence of complement, as heat-inactivated paired samples showed no killing activity. None of the samples showed any effect of neutrophil killing, CFU counts were similar in the presence or absence of neutrophils. There was no difference observed between donors.

The assay was also performed with human leukocytes prepared by erythrocyte lysis. Blood obtained from Donor 1 was used for leukocyte isolation. More serum samples were included in this experiment. wP vaccinated serum was used instead of convalescent serum, because only a small volume was available. Naive and aP vaccinated mouse sera and autologous (Donor 1) and Testline human sera were used. Untreated and heat-inactivated aliquots of each serum were tested, as well as a control without leukocytes for each sample. As high serum-dependent

killing without role of phagocytes was noted in the OPK assay with human neutrophils described above, and the effort was to maximize the role of phagocyte-dependent killing, only 5 % serum was used for opsonization and was diluted to 2,5 % by cell addition. MOI was 1:20, as this was optimal for leukocyte uptake (determined for OPA in Chapter 3.9.).
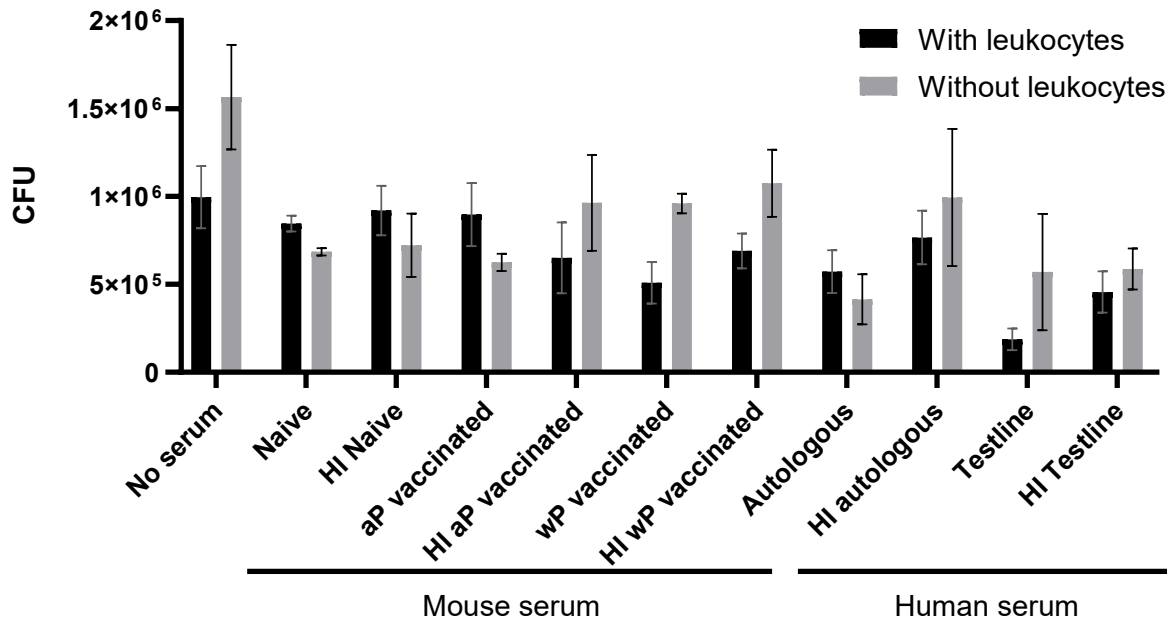

Figure 17 – OPK assay with human leukocytes obtained by erythrocyte lysis – Human leukocytes were obtained from peripheral blood by 3-5 hypotonic lysis steps. Leukocytes were incubated with *Bordetella pertussis* cells at MOI 1:20 and indicated sera at 5 % concentration. The resulting suspension was plated on BG agar and CFU were counted after 5 days.

As seen in Figure 17, addition of leukocytes without any serum resulted in an approximately $5 \cdot 10^5$ CFU reduction (36 %) compared to no serum and no leukocytes control. This shows killing activity of the isolated leukocytes. Also, the resulting CFU reduction compared to control differed according to the serum sample added. Addition of untreated naïve or aP vaccinated mouse serum samples did not lower the CFU further, and no difference was observed between samples with or without leukocytes. For the wP vaccinated serum, there is a clear CFU reduction in the samples with leukocytes compared to samples without leukocytes, by about $5 \cdot 10^5$ (47 %). When wP vaccinated serum sample was compared to the 'no serum, no leukocytes' control, the CFU reduction was 68 %. In the human sera, as observed previously (Figure 16), the effect of complement activation masks the effect of phagocytosis. Some effect of leukocyte killing can be seen in both heat-inactivated samples, and in the untreated Testline serum sample. The Testline serum was the most efficient in killing the bacteria with the addition of leukocytes, reducing the CFU to $1 \cdot 10^4$, 88 % of the no serum, no leukocyte control. As this was performed only once, differences between donors could not be determined.

The leukocytes isolated by erythrocyte lysis proved superior to isolated neutrophils for the OPK assay. Differences between samples with and without phagocytes were observed only in the assay utilizing leukocytes. Isolated neutrophils did not exhibit any killing activity.

3.8. Bead OPA assays

Because isolated human neutrophils failed to exhibit any killing activity, a bead OPA assay was used to test if the lack of killing is caused by lack of uptake. For this assay, Fluoresbrite® YG microspheres (Polysciences, Inc.) were used. The beads were easily detected by flow cytometry, were of a defined size and produced a uniform level of fluorescence. During analysis, it was possible to discriminate cells which had ingested a certain number of beads by fluorescence intensity (Figure 18). Binding of antibodies to beads was unspecific. Any detected effect was not specific against *B. pertussis*, instead it reflected the whole antibody spectrum present. Three methods of obtaining neutrophils were tested.
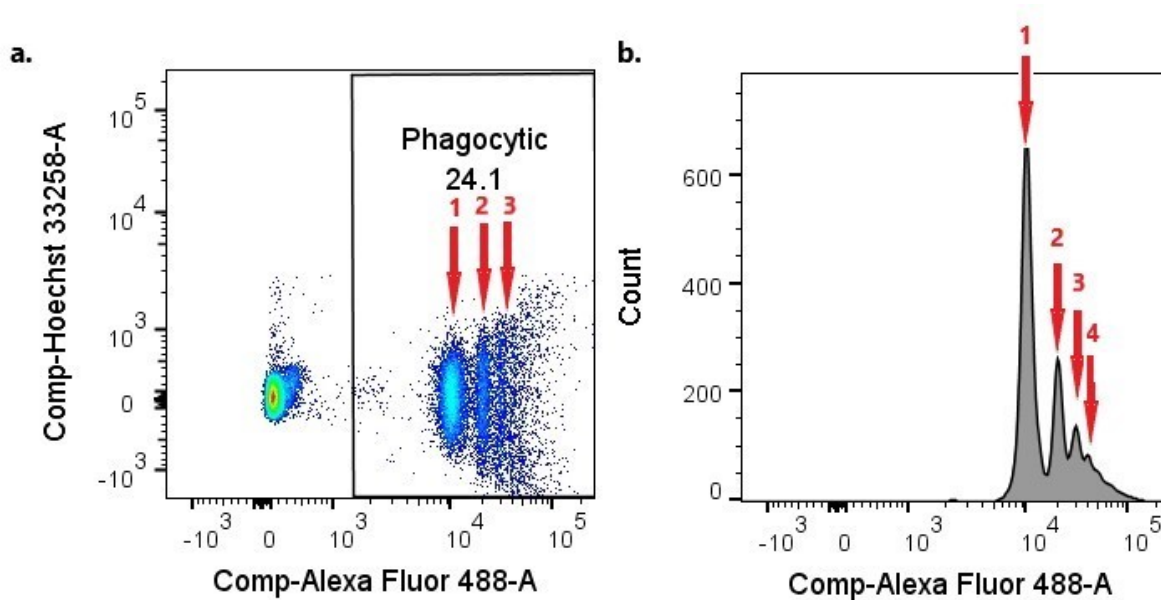

Figure 18 – Ingestion of fluorescent beads by phagocytes – Leukocytes were incubated with serum-opsonized fluorescence labeled beads. Uptake was analyzed by flow cytometry. a) cells containing beads are gated from live cells and shown as a dot plot. b) a histogram of the Phagocytic population is shown. Red arrows indicate peaks corresponding to 1, 2, 3 or 4 ingested beads per cell.

Compensation matrix was calculated by FlowJo software based on single-stained samples and is depicted in Table 10. Gating strategy is depicted in Figure 19.

Table 10 – compensation matrix used in the bead OPA – The table was generated by FlowJo based on single stained samples.

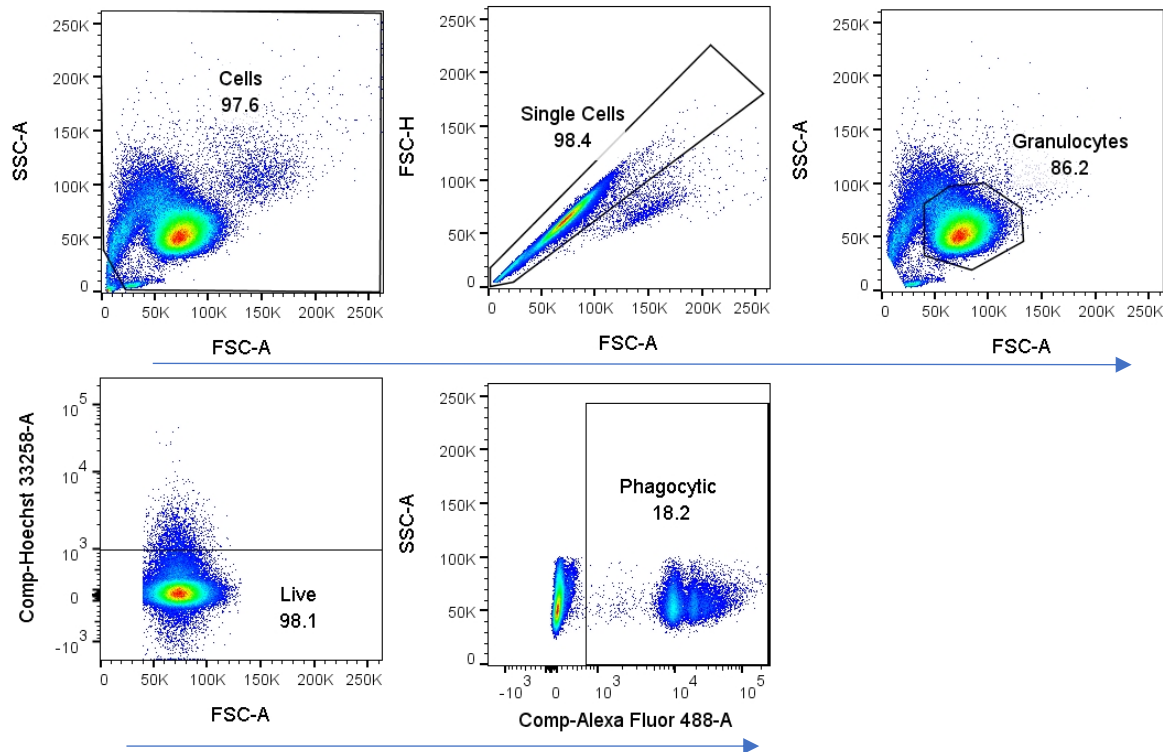|	Bead OPA assay	
	Hoechst-33258	Alexa Fluor-488 (Beads)
Hoechst-33258	100	1,3
Alexa Fluor-488	31	100

Figure 19 – Gating strategy for the bead OPA assay – Cells were prepared by plasma-Percoll isolation and incubated with serum-opsonized fluorescent beads. Only the granulocyte gate was considered.

To determine the optimal cell preparation for the bead OPA, three methods of cell preparation were tested. The 'plasma-Percoll gradient centrifugation' method yields pure neutrophils and was described in Chapter 3.7. The other two methods yield leukocytes. The 'dextran sedimentation' method was a reduced version of the plasma-Percoll gradient centrifugation method. For this technique, the blood was only sedimented by addition of dextran and the leukocyte rich supernatant was aspired, without further gradient centrifugation. The time needed to obtain leukocytes, counted from blood draw to last cell wash, was 2 hours. The 'erythrocyte lysis' method was also described in Chapter 3.7., with percentages of individual cell populations listed for each donor.

The assay variables were different from those of the killing assays. Beads were opsonized in 50 % serum. The obtained cells were incubated with serum-opsonized beads, final serum concentration during incubation being 5 %. MOI of 1:1 was used. Naïve and wP vaccinated mouse sera and autologous human serum were used. Each serum was tested untreated and heat-inactivated. Controls incubated at 4 °C were prepared for each sample. Only granulocytes were analyzed in this assay, as seen in the gating strategy above. The phagocytosis rate was expressed as percentage of cells containing one or more beads.
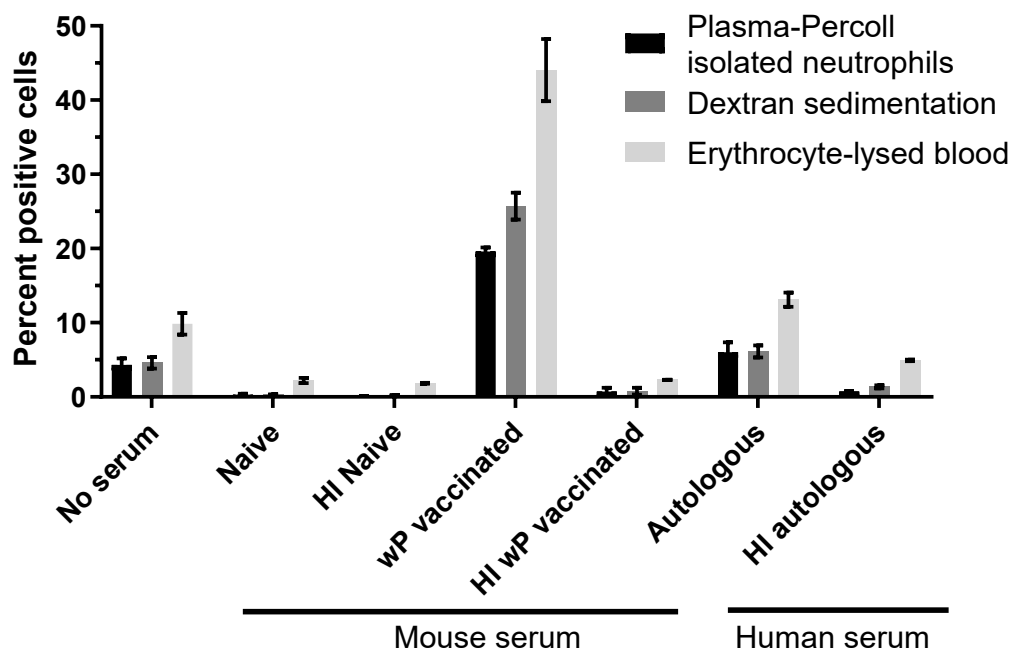

Figure 20 – Comparison of bead OPA assay with cells obtained by three different methods – Serum-opsonized beads were incubated with cells obtained by plasma-Percoll isolated neutrophils, leukocytes obtained by dextran sedimentation or leukocytes obtained by erythrocyte lysis. Uptake of beads was compared by flow cytometry. Results from one experiment are shown, the data being measured in duplicate.

The assay was performed twice, with blood from Donor 1 being used for both experiments. wP vaccinated serum-opsonized beads proved to elicit the highest phagocytosis rates. Neutrophils isolated on plasma-Percoll gradient showed the lowest uptake of such beads. The maximum uptake for isolated neutrophils was 19 %. Leukocytes obtained by dextran sedimentation ingested such beads in 26 % of events. Up to 44 % of leukocytes obtained by erythrocyte lysis contained such beads. Naïve serum and all heat-inactivated sera lowered phagocytosis rates compared to unopsonized control. The differences between naïve and wP vaccinated sera were enhanced when using erythrocyte lysis obtained leukocytes. This technique also provided neutrophils with the highest phagocytosis rates.

3.9. *Bordetella pertussis* OPA assays

Leukocytes acquired by erythrocyte lysis were further used for a Bordetella pertussis (BP) uptake assay. The cells were analyzed by flow cytometry and this approach gives an additional monocyte population to study. Granulocytes and monocytes were separated by side scatter and forward scatter gates. Heat-killed *B. pertussis* expressing mScarlet fluorescent protein were used as target particles. Heat-killed non-fluorescent *B. pertussis* was used as control. Mean fluorescent intensity of the positive population (MFI_{pos}) was used for evaluation of the ingested bacteria.

Heat-killed bacteria were opsonized by 10 % serum, later diluted to 5 % by leukocyte addition. For opsonization, mouse naïve, aP vaccinated and wP vaccinated sera were used, as well as human autologous serum and a control without serum. Different cell to bacteria ratios were tested (1:5, 1:10 and 1:50). After incubation, cells were analyzed by flow cytometry. Granulocyte and monocyte populations were analyzed separately.

Compensation matrix was calculated by FlowJo software based on single-stained samples, and as seen in Table 11, no compensation was necessary. Gating strategy is depicted in Figure 21.

Table 11 – Compensation matrix for the BP OPA assay – The table was generated by FlowJo based on single stained samples.

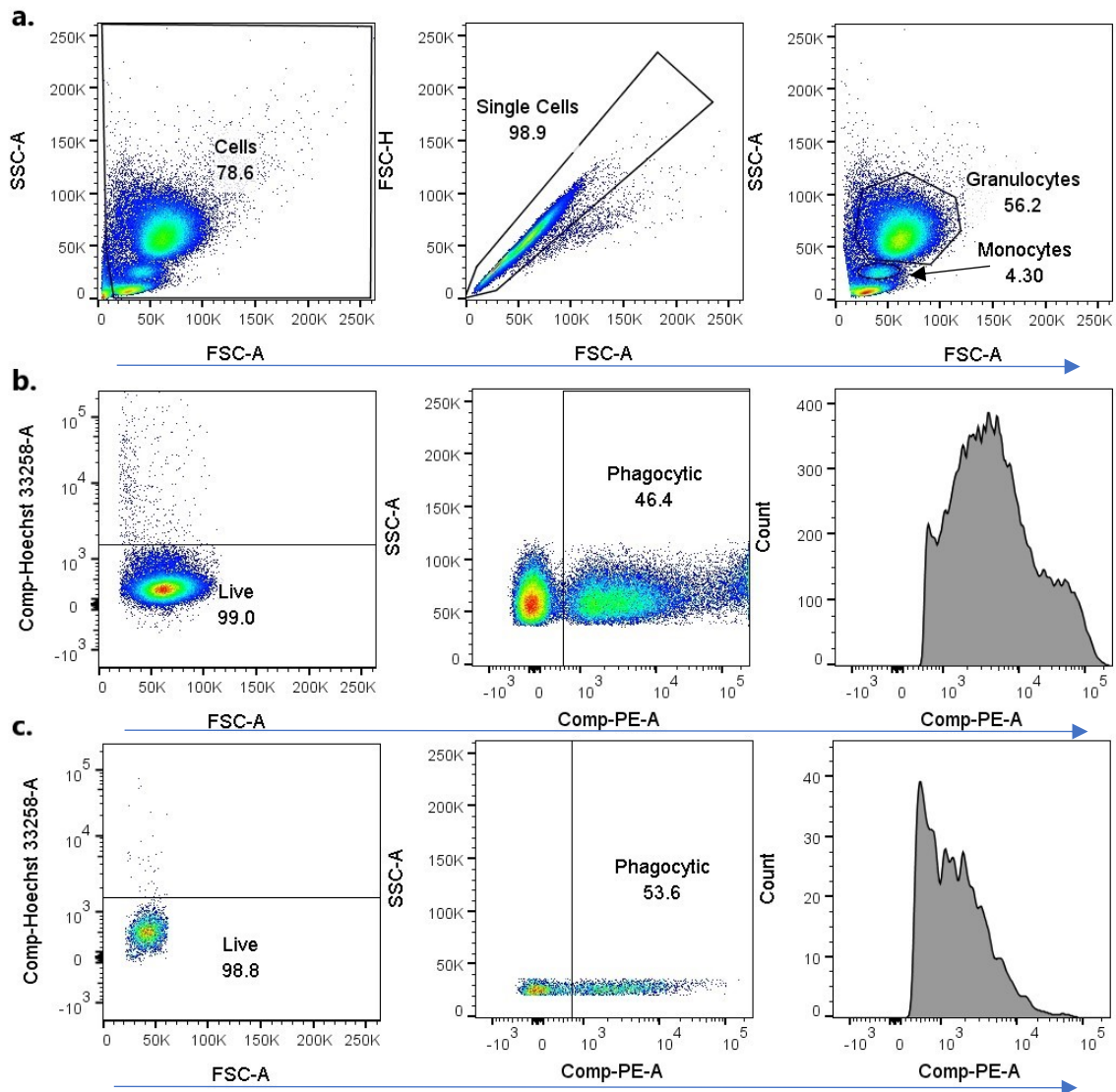| BP OPA assay		
	Hoechst-33258	PE
Hoechst-33258	100	0
PE	0	100

Figure 21 - Gating strategy for the BP OPA assay – Cells were prepared by erythrocyte lysis and incubated with serum-opsonized heat-killed Bordetella pertussis. Part a) shows the gating strategy until cell population selection. Part b) shows viability and phagocytosis gating for granulocytes, part c) for monocytes.

As shown in Figure 22, opsonization with naïve mouse sera gave values comparable to those of unopsonized bacteria. Opsonization with both wP and aP vaccinated sera increased MFI_{pos} values, the aP vaccinated serum more efficiently. The human autologous serum increased the MFI_{pos} values, compared to naïve mouse serum or unopsonized bacteria. Analogously, in the monocyte population, the MFI_{pos} value of naïve mouse serum-opsonized bacteria was the same as MFI_{pos} value of unopsonized bacteria (Figure 22). Opsonization with both wP and aP vaccinated sera increased MFI_{pos} values, here the wP vaccinated serum more efficiently. The untreated human serum did not increase MFI_{pos}, but the heat-inactivated serum did. No clear difference between untreated and heat-inactivated samples was observed.
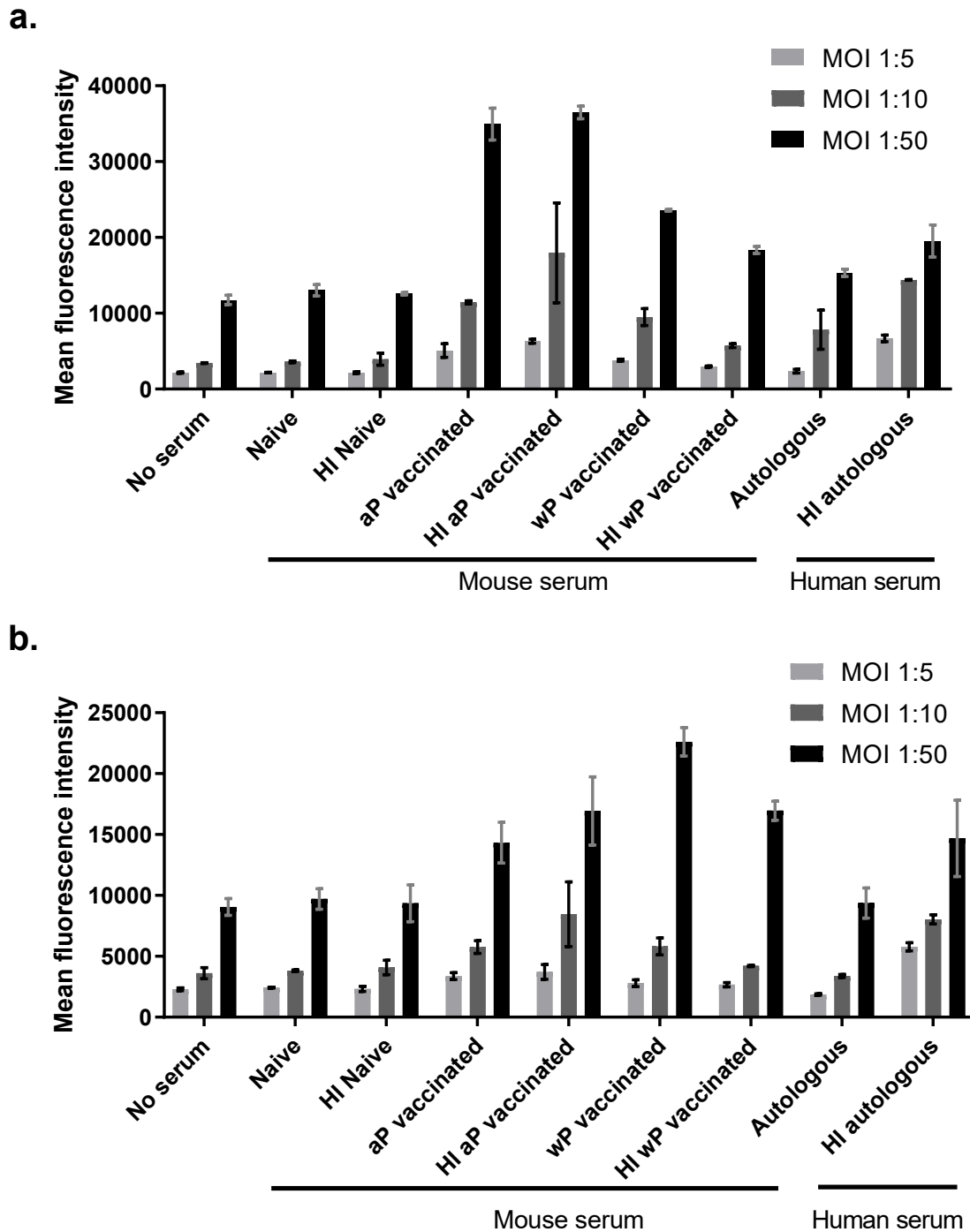
Figure 22 – Bordetella pertussis OPA with leukocytes obtained by erythrocyte lysis – Human leukocytes obtained by erythrocyte lysis were incubated with opsonized fluorescent Bordetella pertussis and uptake of the bacteria was analyzed by flow cytometry. Three different MOI were tested. a, granulocyte population. b, monocyte population.

With MOI 1:5 very little difference between sera were observed. MOI 1:10 highlighted differences between sera better, while keeping low background positivity (unopsonized or

naïve serum-opsonized samples). MOI 1:50 enhanced the differences between sera but had a higher background positivity. MOI of 1:20 was chosen as optimal MOI for further experiments.

3.10. *Bordetella pertussis* OPA with different serum samples

After determining optimal cell preparation and MOI, effects of all available sera were tested in *BP* OPA. Leukocytes isolated by erythrocyte lysis were used at MOI 1:20, heat-killed *Bordetella pertussis* was used as before. All serum samples were used: naïve, aP vaccinated, aP + FIM + ACT vaccinated, wP vaccinated and convalescent mouse sera, also human sera from Donor 1, Donor 3 and the Testline serum. Each serum sample was tested untreated and heat-inactivated. A control without serum was included. Serum concentrations during opsonization were 10 %, diluted to 5 % by phagocyte addition. The assay was performed twice, once with blood from Donor 1, the second time with blood from Donor 2. The amount of ingested bacteria was evaluated separately for granulocyte and monocyte populations.

In the first assay with leukocytes from Donor 1, the naive and aP vaccinated mouse serum opsonization yielded MFI_{pos} values at the level of those when unopsonized bacteria were used. The bacterial uptake was significantly increased in both granulocyte and monocyte populations after opsonization of the bacteria with aP + FIM + ACT vaccinated and wP vaccinated sera. For granulocyte population, convalescent serum also increased uptake (Figure 23). Heat-inactivated sera had lower ability to mediate phagocytosis and, in most samples, yielded lower MFI_{pos} values. The untreated human sera samples showed MFI_{pos} values below the negative sample and unopsonized control and this effect was also observed in the previous experiment (Figure 22).
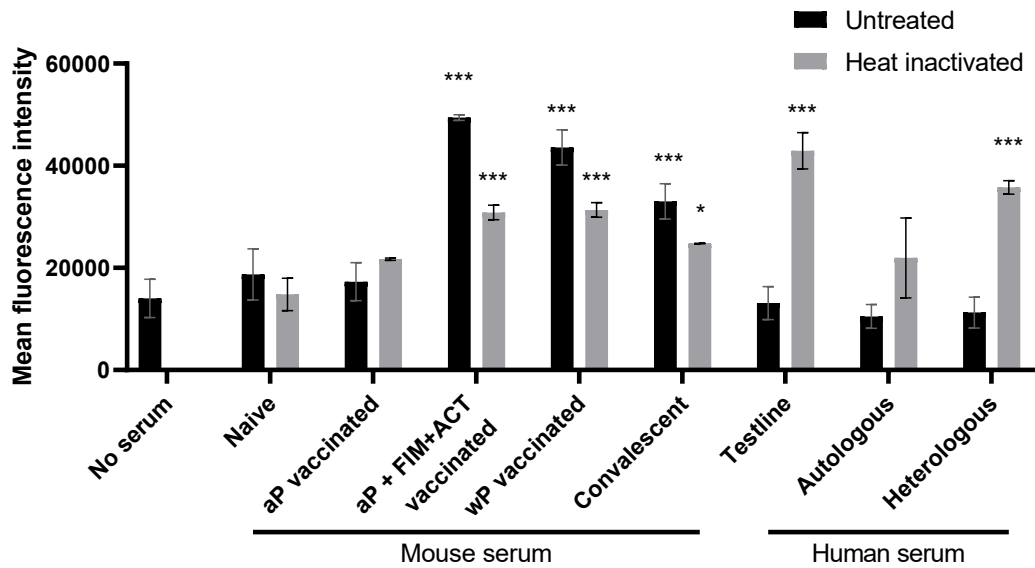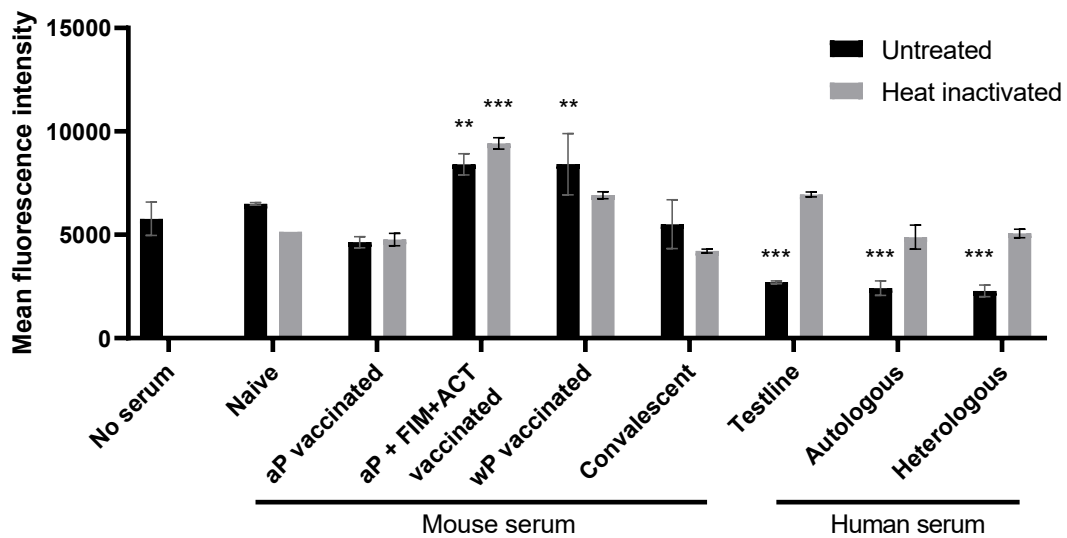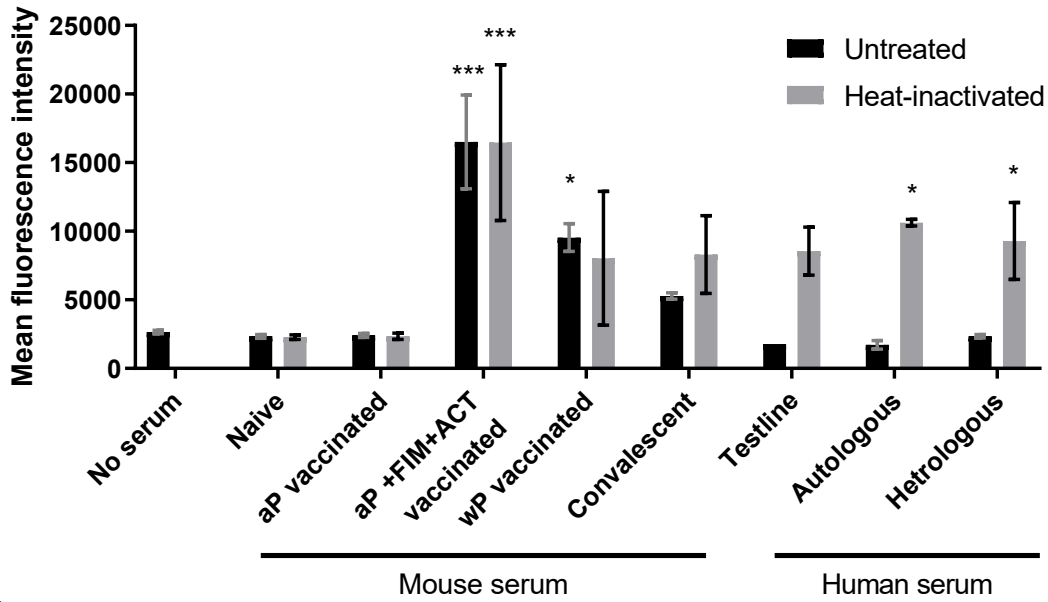
Figure 23 – Bordetella pertussis OPA with leukocytes obtained by erythrocyte lysis, Donor 1 – Human leukocytes obtained by erythrocyte lysis were incubated with opsonized fluorescent Bordetella pertussis and uptake of the bacteria was analyzed by flow cytometry. a) granulocyte population. b) monocyte population. were analyzed by two-way ANOVA followed by Dunett's test for comparison of subgroups to the 'no serum' control. (*, ** and *** represent P values of 0.05, 0.01 and 0.001, respectively).

The same experimental setup was used with blood from Donor 2 (Figure 24). Although the trends of the results were similar, the total MFI_{pos} values were lower. A smaller effect of heat-inactivation was observed in the mouse sera samples. The effect of heat-inactivation on human sera samples was similar as in the previous experiment.
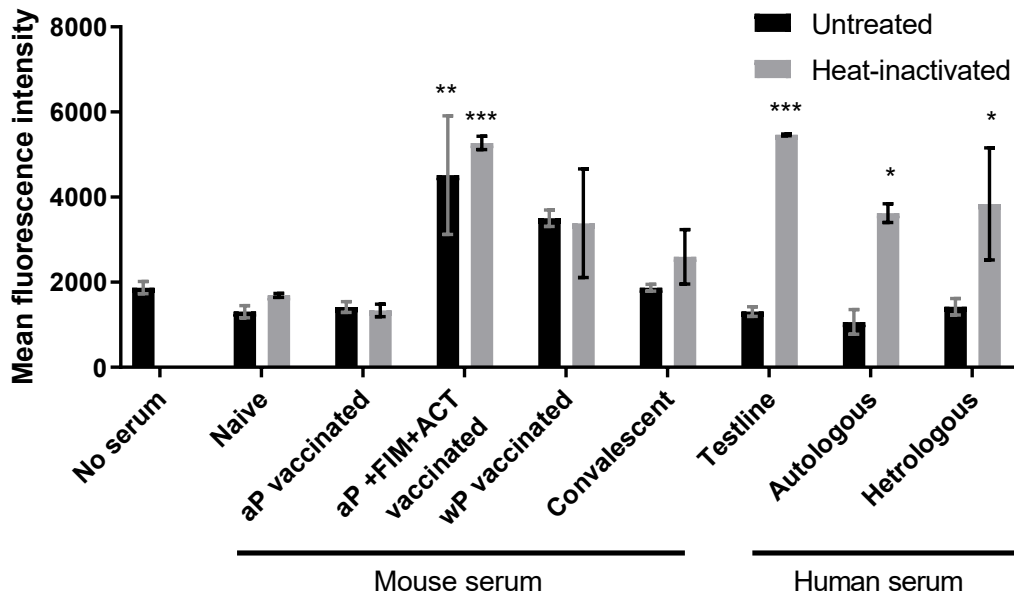
Figure 24 - Bordetella pertussis OPA with leukocytes obtained by erythrocyte lysis, Donor 2 – Human leukocytes obtained by erythrocyte lysis were incubated with opsonized fluorescent Bordetella pertussis and uptake of the bacteria was analyzed by flow cytometry. a, granulocyte population. b, monocyte population. were analyzed by two-way ANOVA followed by Dunett's test for comparison of subgroups to the 'no serum' control. (*, ** and *** represent P values of 0.05, 0.01 and 0.001, respectively).

3.11. *BP* OPA assay with mouse leukocytes

Because the erythrocyte-lysis method is also suitable to prepare mouse leukocytes, this method was compared with the assay using human leukocytes. The main drawback of using mouse cells is the low yield of cells per mouse (about 100 000 phagocytes per mouse).

Ly6G as a specific marker for mouse neutrophils was used to discriminate neutrophil population by flow cytometry. Based on this staining, neutrophils comprised 95 % of the granulocyte population. Single stained samples were used to generate a compensation matrix in FlowJo (Table 12). The gating strategy is shown in Figure 25.

Table 12 – Compensation matrix for BP OPA assay – The table was generated by FlowJo based on single-stained samples

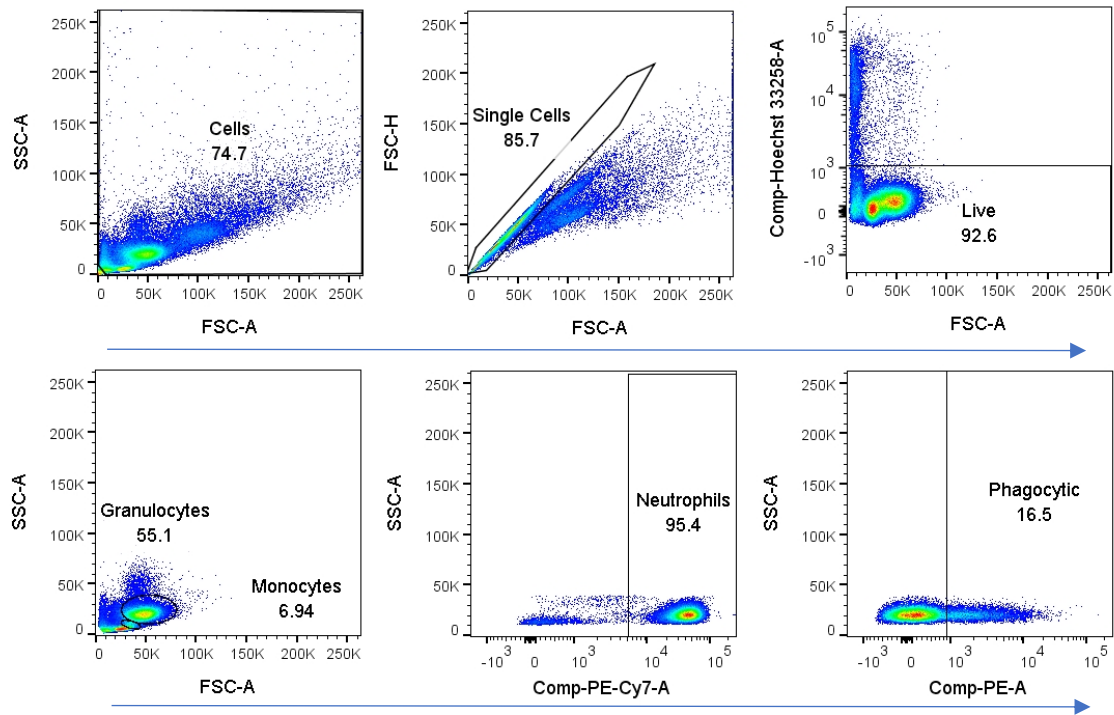| *BP* OPA assay with mouse neutrophils			
	Hoechst-33258	PE-Cy7	PE
Hoechst-33258	100	0	0
PE-Cy7	0	100	0,2
PE	0	14,23	100

Figure 25 – Gating strategy for BP OPA assay with mouse leukocytes – Erythrocytes from mouse blood were lysed by hypotonic NaCl solution and the acquired leukocytes were incubated with serum-opsonized fluorescent Bordetella pertussis. Phagocytic uptake was analyzed by flow cytometry. Only the granulocyte population was analyzed. PE-Cy7-Ly6G was used to label neutrophils.

Only preliminary data were obtained. The setup was chosen as in the OPA assay with human leukocytes: leukocytes were obtained by erythrocyte lysis of fresh mouse blood. Heat-killed mScarlet *B. pertussis* was used as the target particle. Heat-killed non-fluorescent *B. pertussis* was used as a control. Since the cell yield was not high, only mouse sera were tested, and no technical replicates were performed. Mouse naïve, aP vaccinated, aP + FIM + ACT vaccinated, wP vaccinated and convalescent sera were used for opsonization. Untreated and heat-inactivated serum aliquots were compared. Serum concentrations during opsonization were 10 %, diluted to 5 % by phagocyte addition. The MOI used was 1:20.

The MFI_{pos} values obtained from this assay show that both untreated and HI aP + FIM + ACT serum increased phagocytosis in comparison to control. The other untreated sera did not increase phagocytosis compared to control. Heat inactivated naïve, wP vaccinated and convalescent sera exhibited slightly higher MFI_{pos} values than control (Figure 26).
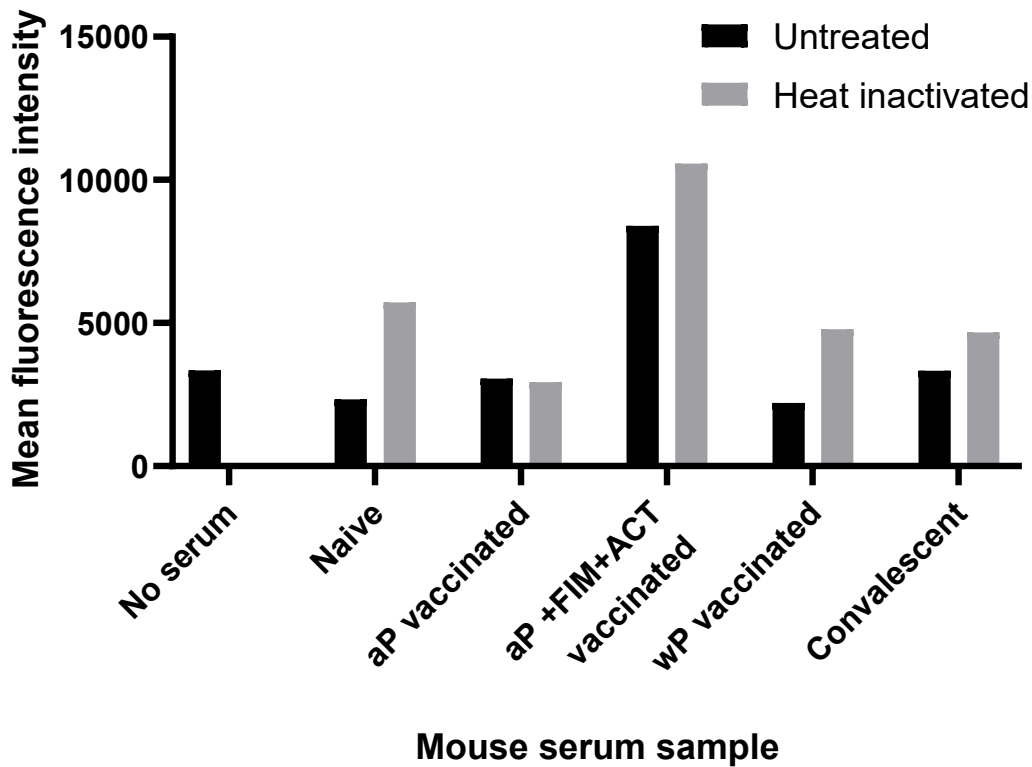
Figure 26 – Bordetella pertussis OPA assay with mouse leukocytes obtained by erythrocyte lysis, granulocyte population – Erythrocytes from mouse blood were lysed by hypotonic NaCl solution and the acquired leukocytes were incubated with serum-opsonized fluorescent Bordetella pertussis. Phagocytic uptake was analyzed by flow cytometry. Only one replicate was performed for each sample.

The data correspond to the results obtained with human leukocytes, but low yields of mouse cells prevent the establishment of the assay.

4. Discussion

For the purpose of developing an opsonophagocytic assay which would provide information about the functional antibody activity against *Bordetella pertussis*, several approaches were tried, including determination of ideal cell type, testing of MOI, serum concentration and other variables.

The serum samples were characterized first. Analysis of serum samples by ELISA was performed, total IgG titers for each serum as well as IgG1 and IgG2a isotype titers for the mouse sera were obtained. The method confirmed that the naïve mouse sample contains a low titer of anti-B. pertussis antibodies. This ruled out any previous contact of the tested mice with the disease. Second, it showed that after immunization or infection, the mice mounted an antibody response to a varying extent. As expected, vaccination with wP vaccine induced a strong antibody response. The antibody titer after infection was higher than after aP immunization but was not as potent as after wP immunization. This may reflect the immunosuppressive effect of virulence factors of the pathogen produced during infection. Human serum titers values were comparable to titers of aP vaccinated (or aP + FIM + ACT vaccinated) mice.

Since a combination of mouse sera and human cells was used in the assays in this thesis, I sought confirmation of suitability of this combination. Murine IgG2a and IgG3 isotypes have been shown to be recognized by and bind to human Fc receptors. Murine IgG1 isotype failed to interact with human Fc receptors (Lubeck et al., 1985). Later studies showed that Fc receptors potentially recognizing IgG1 are polymorphic and can bind to some, but not all receptor variants on human monocytes (Van de Winkel et al., 1987).

Aside from total IgG titers, it was important to determine the IgG2a isotype titer of the mouse sera, as this is relevant to the combination of human cells with mouse sera used in the OPA and OPK assays and the polarization of the response. IgG2a is the mouse isotype that is recognized by human Fc receptors. Also, it activates complement. In contrast, mouse IgG1 is the isotype which is not recognized by human Fc receptors and does not activate complement (Lilienthal et al., 2018; Lubeck et al., 1985; Michaelsen et al., 2004). Aside from aP vaccine, all immunizations and infection induced a higher IgG2a than IgG1 response. A higher IgG2a titer compared to IgG1 or other IgG isotypes after natural infection has been documented (Kirimanjeswara et al., 2003). However, the aP vaccine induced 10x more IgG1 than IgG2a antibodies. Since mouse IgG1 antibodies are associated with a Th2 response, and IgG2a with

a Th1/Th17 response, this is in agreement with vaccine studies, such as those performed on baboons or the studies on humans after vaccination (van der Lee et al., 2018; Warfel et al., 2014).

Agglutinating titer was also determined for each serum. Hellwig et al. (2003) found similar agglutinating titers in their human serum samples as was measured here for human sera. In agreement with that, the agglutinating titers did not correlate with the serum antibody titers (Hellwig et al., 2003). In contrast to human sera, agglutinating titers of mouse sera reflected the total IgG titers.

During assay optimization, three different cell preparations were tested. For the OPK assay, neutrophils isolated by gradient centrifugation from murine bone-marrow did not provide consistent results and proved difficult to be isolated. Although bone-marrow derived neutrophils have been successfully employed to uptake *Pseudomonas aeruginosa* (Monfregola et al., 2012), they did not prove suitable for the OPK. Because of the complications encountered with mouse neutrophils, human neutrophils were chosen as an alternative. Isolation on a plasma-Percoll gradient was tested. Bacterial killing was observed in the OPK assay, but this did not depend on the presence of neutrophils. The bactericidal effect was more likely due to complement activity of the sera. Since there were differences between naïve and immune sera, the most plausible explanation would be an antibody-dependent complement activation mechanism. Human gradient-isolated neutrophils are generally the most commonly used cells (Table 2), but in *Bordetella pertussis* studies, they have been employed mainly in uptake assays. Killing assays with *Bordetella pertussis* as the target bacteria were used by only two groups. Rodriguez et al. (2001) reported no killing in the absence of antibodies, but up to 85 % decrease in CFU when IgG (without complement) was added. Steed et al. (1991) reported lower killing rates, about 20 % CFU reduction even in presence of complement. They used *Escherichia coli* as a control in the killing assay and 99 % of the *E. coli* was killed. Values obtained by our killing assay are comparable to the results of above-mentioned articles, but the effect observed was not dependent on the presence of neutrophils. Complement inactivation abrogated the bactericidal effect in the killing assays I performed, but Rodriguez et al. (2001) obtained high killing rates even with purified antibodies and no complement addition specified. In our hands, human isolated neutrophils did not kill *Bordetella pertussis* despite being used in many opsonophagocytic assays. Their uptake activity was also low, compared to the other tested cell acquisition methods, which are rarer in published results.

I also performed the assay with leukocytes obtained by erythrocyte lysis of fresh blood. Because of the high serum bactericidal activity observed in the assay with isolated neutrophils, serum concentration during opsonization and killing was lowered. Even so, the percentages of killing were high. Leukocytes were used for opsonophagocytic assays with *Bordetella pertussis* only in an uptake assay (Aase et al., 2007). In the killing assay for this thesis, they showed moderate killing of unopsonized bacteria and a good distinction between tested sera. However, the assays should be repeated in order to obtain reproducible results.

The bead OPA was then used to determine if the lack of neutrophil-dependent killing is caused by lack of uptake. I compared three isolation methods, and this proved to be an important comparison. It showed that the erythrocyte-lysis technique provided the most active cells when considering phagocytic uptake. The bead uptake of plasma-Percoll isolated neutrophils was low. Due to unspecific antibody binding to beads, any detected effect is not specific against *B. pertussis*, instead it reflects the whole antibody spectrum present. Also, the uptake of beads was dependent on complement, because heat-inactivation of sera abrogated any ingestion. The isolated neutrophils showed the lowest uptake capacity, the leukocytes gained by dextran sedimentation showed only slightly higher uptake. The uptake by leukocytes obtained by erythrocyte lysis was the highest (45 % of cell containing particles) in presence of immune sera, while it was very low (2 % of cell containing particles) after opsonization with the naïve serum.

The findings from the bead OPA were implemented to establish the *BP* OPA. First, optimal MOI was determined to maximize differences between sera. Finally, test sera were analyzed in the *BP* OPA using leukocytes from two donors. The assay discriminated naive from immunized and convalescent mouse sera. It also differentiated between healthy human serum and positive control Testline serum, but only in the heat-inactivated samples. Due to high bactericidal effect seen in the killing assay (Chapter 3.7.), it is highly possible that complement-mediated lysis reduced the number of bacteria present in the suspension, thus mimicking a situation with a much lower MOI. Aase et al. used the same cell type for their *Bordetella pertussis* opsonophagocytic uptake assay but evaluated respiratory burst instead of internalized bacteria. In their assay, unopsonized bacteria did not trigger a respiratory burst, opsonized bacteria did (Aase et al., 2007). Other authors who evaluated uptake of *B. pertussis* used different cell types and provided conflicting results. Opsonophagocytosis by human neutrophils has been shown to differentiate between control, symptomatic and convalescent human sera, alike to our assay (Hovingh et al., 2018). Also, it has been shown that uptake does not take place as efficiently

in absence of opsonizing antibodies (Hellwig et al., 2003). Others have shown that opsonization with convalescent serum decreases uptake by neutrophils, which is most commonly explained by decreasing attachment of the bacteria to the phagocytes (Mobberley-Schuman et al., 2003; Weingart and Weiss, 2000). Such a decrease was not observed in our assay. Results suggesting that opsonization by serum from immunized individuals has no role in opsonophagocytosis also have been published (Stefanelli et al., 2002; Christine L. Weingart et al., 2000). As there is no consensus on the role of opsonizing antibodies in uptake, it would be helpful to test other phagocytosis evaluation approaches in the future, such as respiratory burst measurements.

I also tested whether human cells reflect the situation using mouse cells. Here, the erythrocyte lysis method provided low yield of cells. Although only preliminary data were acquired, the overall trends suggest some differences in uptake between the human cells and mouse cells. aP + FIM + ACT vaccinated serum increased uptake in both mouse and human cells. In the mouse assay opsonization of *B. pertussis* cells with wP vaccinated and convalescent serum did not increase phagocytosis, which is in contrast with the assays performed on human phagocytes. The mouse neutrophils were not suitable for OPA assays in our hands, although they were used successfully for uptake assays by other groups (Hellwig et al., 2001; Maini et al., 2018).

In general, the results of the individual assays distinguish immune sera from naïve, although the aP vaccinated serum rarely induced higher uptake or killing than naïve serum. The mouse leukocyte OPA preliminary results are the only exception. The killing assay indicates an important proportion of killing dependent on complement. The *BP* OPA assays did not show such an important role of complement for uptake, in agreement with data of Hellwig et al. (2001). In contrast, uptake in the bead OPA was abrogated with heat-inactivation of sera, suggesting a different mechanism for the uptake of the beads. The main effect of complement observed in the *BP* OPA assays was in the case of human sera, where complement mediated-lysis could be responsible for the low uptake of untreated human serum-opsonized bacteria. It appears that human serum samples would be better used heat-inactivated, as in many published assays, or at a lower concentration to dilute complement components.

5. Conclusion

This thesis focused on development of an opsonophagocytosis assay for measurement of functional antibody activity against *Bordetella pertussis*. My goals were to optimize the assay conditions and subsequently test available mouse and human sera.

First, antibody titers of the available sera were measured by the ELISA method, quantifying total IgG titers, IgG1 and IgG2a isotype titers for mouse sera and total IgG titers were measured for human sera. Also agglutinating titers were measured for all serum samples.

Two isolation methods were tested for mouse neutrophil acquisition and three cell isolation methods for human neutrophil acquisition. Activity of the cells in opsonophagocytic uptake assays was evaluated, first with polystyrene beads as target particles, later with *Bordetella pertussis* as target particles. Leukocytes obtained by erythrocyte lysis proved to be the most suitable for the opsonophagocytic assays. Optimal multiplicity of infection was determined.

The OPK results were not sufficiently reproduced and the results did not discriminate between samples and controls sufficiently. Only serum from wP vaccinated mice and the Testline serum mediated an increase in opsonophagocytic killing.

The OPA assay was established, and the serum samples were evaluated in an opsonophagocytic uptake assay. All immune mouse sera, except for the aP vaccinated mouse serum, increased phagocytic uptake. The naïve mouse serum and aP vaccinated mouse serum did not increase phagocytic uptake. All human sera increased phagocytic uptake, but induced lysis of opsonized bacteria prior to the beginning of the assay when intrinsic complement was present.

6. Literature

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