Monitoring the immune responses to vaccination and pertussis: bordetella pertussis and beyond

Diks, A.M.

Chapter 3.4

Distinct cellular kinetics in participants protected from colonization upon *B. pertussis* challenge

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Manuscript in preparation

Summary
The authors apply high-dimensional flow cytometry to monitor the cellular immune response in a human *Bordetella pertussis* challenge model. These cellular studies show that participants protected from colonization have a distinct early cellular immune response, likely reflecting effective immunological memory.

Abstract
Background: Limited data is available on the mechanisms of protection against colonization with *Bordetella pertussis* in humans.

Methods: Cellular responses to *Bordetella pertussis* challenge were monitored longitudinally using high-dimensional EuroFlow-based flow cytometry, allowing quantitative detection of >250 different immune cell subsets in blood.

Results: Participants who were protected from colonization showed different early cellular responses compared to colonized participants. Especially prominent for non-colonized participants were the early expansion of (CD36-) non classical monocytes at day 1 (d1), NK cells (d3), follicular T helper cells (d1/d3) and plasma cells (d3). Plasma cell expansion at d3 correlated negatively with the CFU load at d7 and d9 post-challenge. Participants that seroconverted showed increased plasma cell maturation at d11-14.

Conclusion: These early cellular immune responses following experimental infection can now be further characterized and potentially linked to an efficient mucosal immune response, preventing colonization. Ultimately, their presence may be used to evaluate whether new *Bordetella pertussis* vaccine candidates are protective against *Bordetella pertussis* colonization.

Introduction

The introduction of vaccines has significantly reduced the amount of infectious disease-related deaths.[1] However, many vaccines are based on empirical results, and the mechanisms of protection and underlying immunological processes are not completely understood. For vaccines/diseases for which no clear correlate of protection is defined, as is the case for pertussis, increased insight into the immunological processes associated with infection may be an important step forward. Pertussis is a disease of the respiratory tract, caused by the bacterium *Bordetella pertussis* (Bp). Infection with Bp occurs via respiratory droplets. Although the whole cell pertussis (wP) vaccine and the acellular pertussis (aP) vaccine are known to protect against disease, the incidence of pertussis has been rising in the past decennia.[2] Several animal models have been established to study pertussis infection and vaccination. These models resulted in valuable insights into the different immune responses induced by aP and wP vaccination, new vaccine candidates, different routes of administration, and infection.[3-5] In baboons, aP vaccination induced a T helper (Th) 1/Th2 response, protecting animals from severe disease, but did not prevent colonization and transmission to naive vaccinated animals.[3] In contrast, natural infection with Bp and -to a lesser extend- wP vaccination induced a Th1/Th17 memory response. Moreover, wP vaccinated animals were protected from severe disease, and although not protected against colonization, they cleared the infection significantly quicker than aP-vaccinated and naive animals. Lastly, previously infected animals were protected from colonization and disease.[3] The question remains to what extent animal models resemble the course of Bp infection and the accompanying immune response in humans. This topic can only be addressed in human vaccination and challenge studies.

Data on humans in the pre-pertussis vaccine era indicated that although natural infection does not result in complete lifelong protection, reinfection is much milder than primary infection.[6] Moreover, protection after natural infection was estimated to last longer compared to aP or wP vaccine-induced protection.[6] This implies that despite high vaccination coverage, the carriage and transmission of pertussis persist, which may explain the reported resurgence of pertussis in the last decades.[7-9] Improved protection against transmission is an important aspect for the development of future vaccines/vaccination strategies against pertussis.

Currently, modifications of existing pertussis vaccines as well as new vaccine candidates are being investigated.[4] Yet limited data is available on the mechanisms and immune signatures associated with protection against colonization. Moreover, it is difficult to extrapolate data from intramuscular vaccination to other routes of administration, such as intranasal delivery, which more resembles the 'natural way' of Bp encounter. For pertussis, the live-attenuated BPZE1 vaccine -which is delivered intranasally- is currently one of the most advanced vaccine candidates in clinical development also aiming to reduce the transmission of pertussis.[4,10]

In 2017, a human challenge model for Bp (controlled bacterial infection) was established at the National Health Institute for Health Research Clinical Research Facility (CRF) Southampton, UK by the University of Southampton as part of the IMI-2 PERISCOPE Consortium.[11,12] In this model, asymptomatic colonization of participants can be established in a safe way. A major advantage of using this human challenge model is that one can define more clearly whether a participant is protected against colonization by measuring serum antibodies and culturing bacteria from throat and nasal samples. Moreover, in contrast to sampling from pertussis patients, the exact moment of infection is known, and the synchronized sampling in multiple participants enables us to find shared patterns arising after bacterial challenge. The analysis of the cellular immune response following bacterial challenge may yield new insights in the cellular kinetics important for early removal of bacteria from the mucosal layers and protection against colonization. Comparison of similarities and differences between the immune responses launched after vaccination or bacterial challenge may help to identify why current pertussis vaccines do not fully protect against infection and what should be changed to induce protection against carriage. Identification of immune signatures associated with protection against colonization may be of importance in the evaluation of vaccines that aim at reduction/prevention of carriage and transmission. In this exploratory study, we monitored the cellular kinetics in humans after intranasal challenge with Bp. All participants were wP-primed and had no known recent contact with Bp. We monitored the immune responses in these participants and identified cellular changes that were unique for participants protected from colonization.

Results

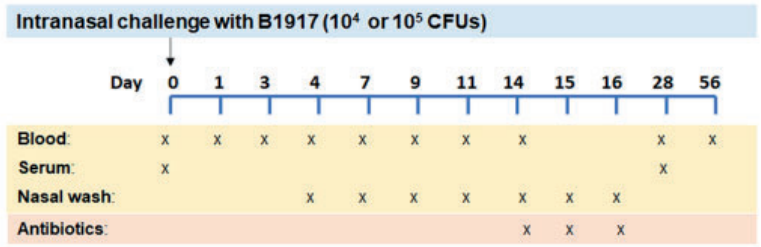
10/15 participants colonized after challenge with B1917 stain

Samples from 15 participants were collected between November 2017 and September 2018. Five participants were challenged intranasally with 10^4 colony forming units (CFU) and ten participants were challenged intranasally with 10^5 CFU. As reported previously, participants challenged with 10^5 CFU more frequently reported mild symptoms of cough, rhinorrhea and nasal congestion than participants challenged with 10^4 CFU.[12] None of the participants developed pertussis disease or required rescue medication. No serious adverse events were reported. In the here-presented study, no differences in immune cell kinetics were found between participants challenged with 10^4 or 10^5 CFU, unless indicated specifically. One participant withdrew from the study after day (d) 14, and one participant withdrew after d28, both not related to the study. Samples collected until the moment of withdrawal were used.

To determine the colonization status after challenge, nasal washes were performed from d4 onwards as described previously (**Fig 1A-B**).[12] Challenge with 10^4 CFU and 10^5 CFU resulted in Bp positive culture in 2/5 and 8/10 participants, respectively. Of the 10 colonized participants, four participants showed low-density colonization (defined as <1000 CFU/mL at any time point during the study), and six participants showed high-density colonization (>1000 CFU/mL at

any time point during the study). In further analyses, we divided the participants based on colonization and seroconversion status and searched for unique immune signatures associated with these readouts.

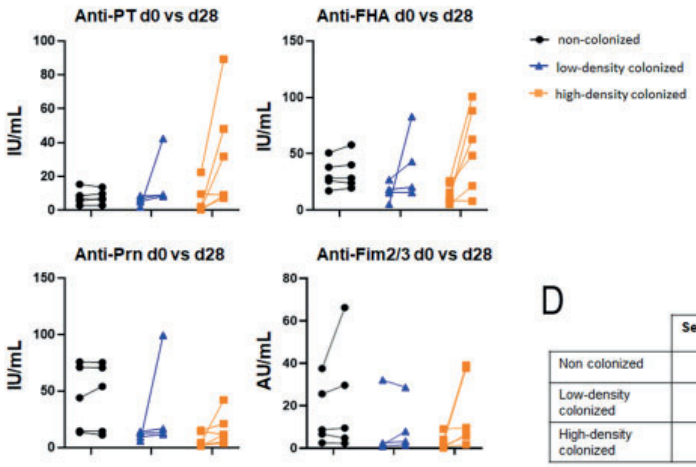
A



B

	Challenge dose	Nasal Wash-derived *Bp* Culture (CFU/mL) Day 4	Day 7	Day 9	Day 11	Day 14	Day 15	Day 16	> Day 16
Non-colonized participants									
ID.01	10^4	0	0	0	0	0	0	0	N/A
ID.02	10^4	0	0	0	0	0	0	0	N/A
ID.03	10^4	0	0	0	0	0	0	0	N/A
ID.04	10^5	0	0	0	0	0	0	0	N/A
ID.05	10^5	0	0	0	0	0	0	0	N/A
Low-density colonized participants									
ID.06	10^5	6	0	12	180	12	0	0	N/A
ID.07	10^5	168	534	48	72	18	0	0	N/A
ID.08	10^5	0	114	0	0	0	0	0	N/A
ID.09	10^5	0	180	360	60	330	24		N/A
High-density colonized participants									
ID.10	10^4	0	288	132	5508	444	0	0	N/A
ID.11	10^4	18	426	2766	2952	3402	0	0	N/A
ID.12	10^5	48	2142	294	7920	39360	0	0	N/A
ID.13	10^5	48	540	6	>60000	>60000	366	0	N/A
ID.14	10^5	0	1140	14460	60000	60000	5088	1656	0
ID.15	10^5	0	13260	24300	13620	2640	6	72	0

C



D

	Seroconversion
Non colonized	0/5
Low-density colonized	1/4
High-density colonized	5/6

Figure 1. Study and cohort description and clinical readout. A. Overview of intervention and sampling time points used in this study, including the mandatory antibiotics treatment for all participants at d14, d15 and d16 after bacterial challenge. B. Overview of participants clustered based on the clinical readout (colonized/not colonized and degree of colonization: no colonization, low-density colonization, and high-density colonization), based on the cultures derived from nasal washes and the anti-PT IgG values at baseline and 1 month after challenge. C. Ag-specific serum IgG levels at baseline and 28 days after challenge, as evaluated by multiplex immunoassay. Data are arranged according to degree of colonization. D. Number of non-colonized, low-density colonized, and high-density colonized participants that seroconverted. PT= pertussis toxin, FHA= filamentous hemagglutinin, Prn= pertactin, Fim2/3= Fimbriae 2 and 3.

Ag-specific serum IgG levels (against PT, FHA, Prn and Fim2/3) were determined at baseline and d28 (**Fig 1C**). Comparison of baseline Ag-specific serum IgG levels of the 15 participants evaluated in this study versus all participants included in the overarching bacterial challenge study showed no differences (data not shown). Six participants showed signs of seroconversion (one low-density and five high-density colonized participants), non-colonized participants showed no sign of seroconversion (**Fig 1D**). On top of increased anti-PT IgG serum levels, colonized participants also showed generally higher increases in other Ag-specific Igs than non-colonized participants. As only an >2-fold increase of anti-PT serum IgG was used as cut-off for seroconversion, we also investigated if any participant had an >2-fold increase for another antigen, but not for PT. This was not observed. Lastly, the absolute increase in Ag-specific serum IgG was most prominent in high-density colonized participants, especially for anti-PT and anti-FHA IgG.

Fluctuations in circulating innate immune cells after bacterial challenge

During natural encounter or challenge, the bacterium itself triggers innate immunity at the site of infection (mucosal surfaces). Thus, a fast (and local) innate response may be important to control Bp directly upon encounter. As recruitment of innate cells to and from tissues may be detected in the blood early after challenge, we evaluated the cellular changes of innate immune cell subsets after bacterial challenge.

Early after challenge, total circulating leukocyte numbers (expressed as ratio of baseline) did not change. However, at d11, d28 and d56 post-challenge, circulating leukocyte numbers decreased (**Fig 2A**). Circulating neutrophils decreased significantly at d1, d4 and d11 post-challenge (ratio of baseline) (**Fig 2B**). Interestingly, when dividing into mature and immature neutrophils, this decrease was only observed for mature neutrophils (not shown). Circulating eosinophils decreased at d3, d4, d28 and d56 post-challenge (**Fig 2C**). The most prominent changes were observed in circulating monocyte populations. Monocytes mature from classical monocytes (cMo), via intermediate (iMo) to nonclassical monocytes (ncMo) and can be further subdivided into different functional subsets or activation stages.[13,14] Fluctuations in total cMo were limited, with only a minor reduction in circulating cMo at d1, d11 and d28 (data not shown). However, within cMo subsets, decreased numbers (ratio of baseline) were observed for CD62L+ cMo at d1, d4, d9, d11, d14, d28 and d56 post-challenge, whereas increased numbers (ratio of baseline) were observed for CD62L- cMo at d1, d4, d7, d9, d28 and d56 post-challenge (**Fig 2D+E**). CD62L, also known as L-selectin, is shed upon activation and CD62L- cMo are considered activated and possibly more mature.[15] Moreover, an increase in intermediate monocytes (iMo) was observed at d3 and d4 post-challenge (ratio of baseline) (**Fig 2F**). No consistent early fluctuations were observed for ncMo, although their numbers decreased at d11 post-challenge (ratio of baseline) (**Fig 2G**). Within the dendritic cell (DC) compartment no early consistent changes were observed, although both plasmacytoid DCs (pDC) and myeloid DCs (mDC) showed a decrease in cells from d28

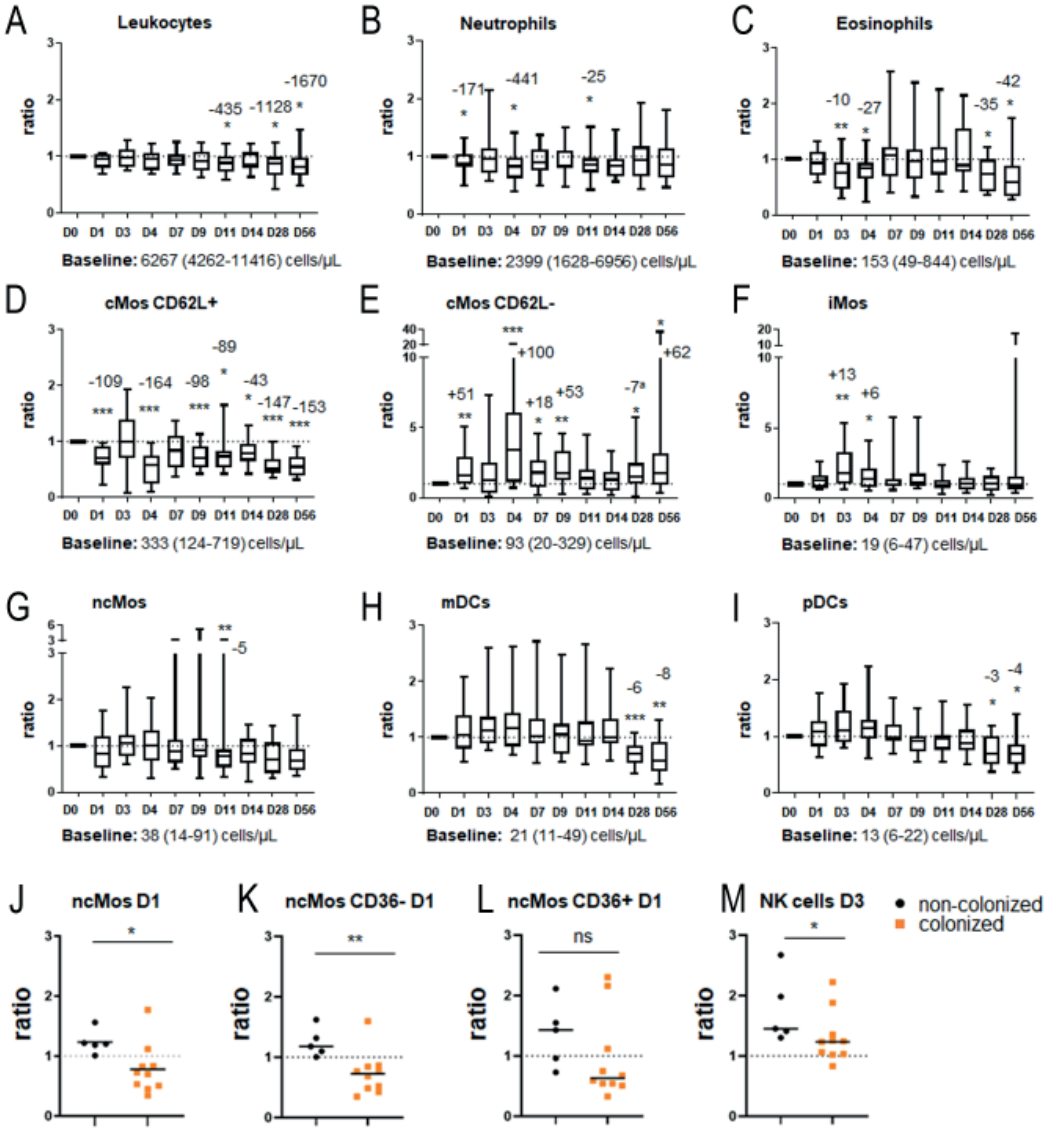


Figure 2. Fluctuations of cell numbers in the innate immune cell compartment after bacterial challenge. Boxplots (median, Q1, Q3, min-max) showing the kinetics of major innate immune cell subsets (A-I) expressed as ratio compared to baseline. N=15. Dashed line indicates a ratio of 1.0 (baseline value). Baseline cell counts, median (min-max) in cells/µl are indicated below each graph. Longitudinal changes were evaluated using Wilcoxon test and ratio of baseline. In case of significant differences in ratio compared to baseline, the median increase or decrease in cells/µl is indicated on top of the bar. (J-M) Increase or decrease of cell numbers expressed as ratio compared to baseline in colonized and non-colonized participants. N=15. Statistical test performed; Mann-Whitney on ratio of baseline. * Indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001. D=days post-challenge. cMo= classical monocyte. iMo= intermediate monocyte. ncMo= non-classical monocyte. mDC= myeloid dendritic cell. pDC = plasmacytoid dendritic cell. a Although in panel E the ratio versus baseline was increased, the median cell count at d28 vs median cell count at baseline was lowered (-7 cells/ul).

onwards (**Fig 2H+I**).

To investigate whether the kinetics of innate immune cells are associated with colonization, we compared the numbers of circulating innate immune cells in

participants who were colonized and non-colonized. No consistent differences between colonized and non-colonized participants were observed for leukocytes, eosinophils, iMo, cMo and dendritic cells. For circulating neutrophils, the decrease in mature neutrophils was visible as a trend in non-colonized individuals at d1 (p=0.125), d4 (p=0.0625), and d11 (p=0.0625), but was less prominent in colonized individuals. An increase in circulating ncMo (ratio of baseline), especially CD36- ncMo, was found at d1 post-challenge in non-colonized participants (**Fig 2J-L**), while these were decreased in colonized participants. Finally, at d3 post-challenge, non-colonized participants had higher NK-cell expansion (ratio of baseline) than colonized participants (**Fig 2M**).

Next, we divided the colonized participants based on colonization density (low- or high-density colonization). Here, we found that the decrease in (especially CD36-) ncMo was most prominent for low-density colonized participants (**Suppl. Fig 1A-C**). cMo maturation (represented by shedding of CD62L) did not differ between low- and high-density colonized participants (data not shown). However, we did observe a decrease in a circulating mDC subset in low-density colonized participants (CD1c+ CD14dim mDC, **Suppl. Fig 1D**). Lastly, NK cells in high-density colonized participants did not expand, whereas the NK cell expansion in low-density colonized participants -to some extent- resembled that of the non-colonized participants (**Suppl. Fig 1E**).

To summarize: we observed fluctuations in innate cell numbers in the early days after bacterial challenge. Decreased cell numbers may indicate migration of cells into the tissue, and the shedding of CD62L may indicate a shift towards a more activated phenotype in the cMo compartment. Cellular kinetics differed between non-colonized and colonized participants, and between high- and low-density colonized participants. The colonization density did not reflect the magnitude of cellular changes, as these were usually more prominent in low-density colonized participants.

Limited fluctuations of T-cell populations post-challenge

Activation of T cells is required for cellular immunity and the activation of humoral immunity by providing T-cell help to B cells in the germinal center reaction. As there are different T-cell responses generated after vaccination with aP or wP and (natural) infection [16,17], we set out to determine changes in circulating T cells after experimental exposure to Bp.

Within circulating total T cells, CD4 T cells, CD4 T helper (Th) and regulatory T cells, no changes occurred until d28 and d56, when a significant decrease compared to baseline was observed (**Fig 3A-C**). However, an expansion of follicular T helper (FTH) cells was observed at d3 post-challenge (ratio of baseline) (**Fig 3D**). This expansion of FTHs was not polarized to any specific FTH cell type. Additionally, no consistent changes were observed in CD4 Th cell fluctuations (**Fig 3E-I**) or Th cell maturation/activation.

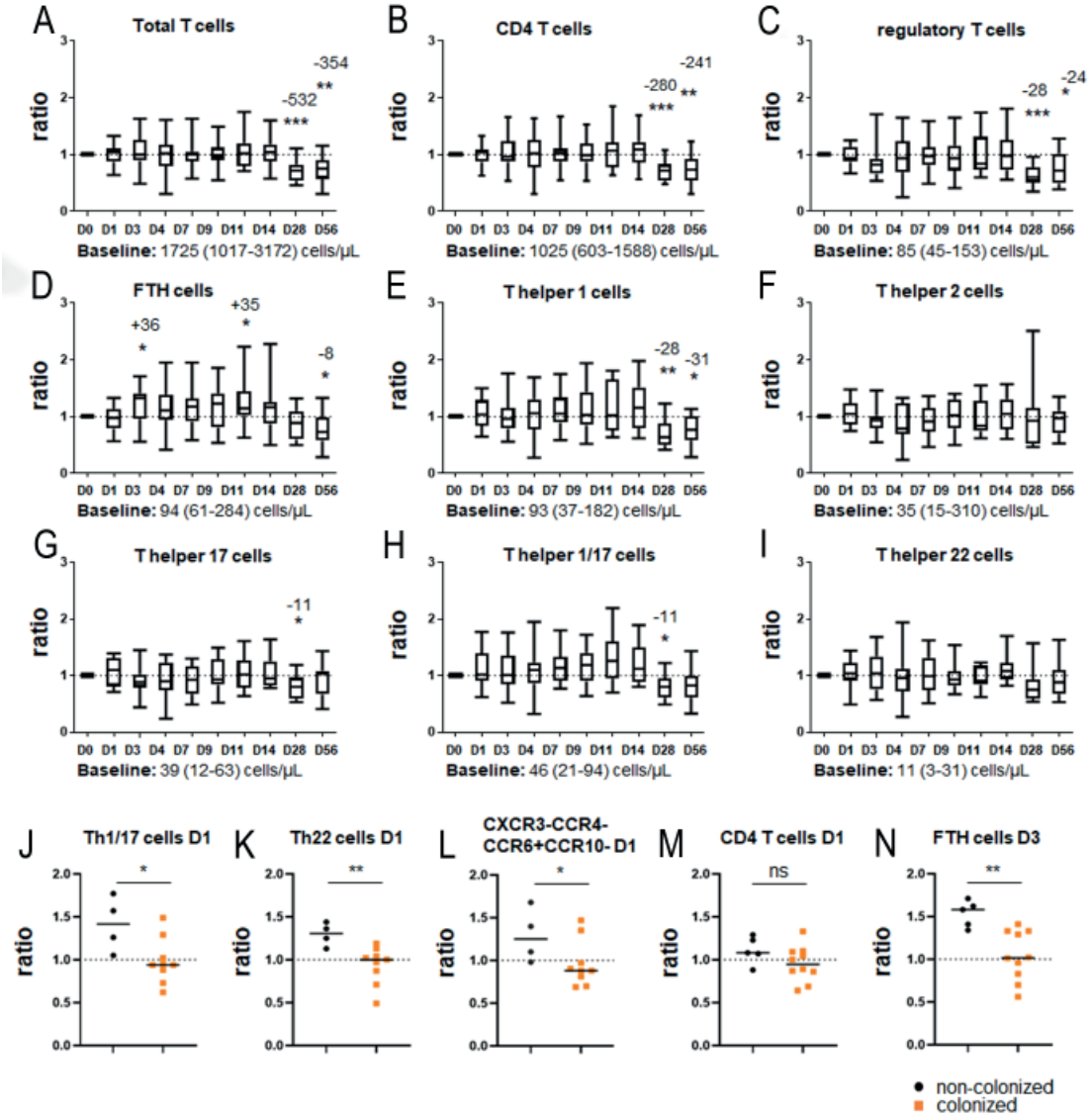


Figure 3. Fluctuations of cell numbers in the T- cell compartment after bacterial challenge. Boxplots (median, Q1, Q3, min-max) showing the kinetics of major innate immune cell subsets (A-I) expressed as ratio compared to baseline. N=15. Dashed line indicates a ratio of 1.0 (baseline value). Baseline cell counts, median (min-max) in cells/µl are indicated below each graph. Longitudinal changes were evaluated using Wilcoxon test and ratio of baseline. In case of significant differences in ratio compared to baseline, the median increase or decrease in cells/µl is indicated on top of the bar. (J-N) Increase or decrease of cell numbers expressed as ratio compared to baseline in colonized and non-colonized participants. N=13 (J-L) or N=15 (M, N). Statistical test performed; Mann-Whitney on ratio of baseline. * Indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$. D= Days post-challenge. FTH= follicular T helper cells, Th= T helper cell, CXCR3-CCR4-CCR6+CCR10- = recently defined Th subset with phenotype CXCR3-CCR4-CCR6+CCR10-.

When grouping the participants based on colonization status, three different CD4 Th subsets increased significantly in non-colonized participants (ratio of baseline) compared to colonized participants at d1 post-challenge (**Fig 3J-L**). A small expansion in Th1/17 cells was observed at d1 post-challenge. Additionally, we found an expansion of Th22 cells and of a recently defined Th subset (phenotype:

CXCR3-CCR4-CCR6+CCR10-) at d1 in non-colonized participants.[18] The difference between colonized and non-colonized participants was not fully explained by total CD4 T-cell kinetics (**Fig 3M**). An increase in total FTH cells at d3 was primarily found in non-colonized participants (**Fig 3N**). Due to technical and biological reasons (missing antibody in a surface stain 'master mix' and/or a CD45RA polymorphism [19]), at this time point FTH subsets could only be defined in 9/15 participants. Nevertheless, we observed a trend towards increased naive FTHs, Th1-like, Th1/17-like and Th17-like FTHs at d3 post-challenge in non-colonized participants. This increase was significant for Th2-like FTH cells (**Suppl. Fig 2**). When looking at the impact of colonization density, comparison between non-colonized and low-density colonized participants resulted in significant differences for Th-cell subsets at d1 (**Suppl. Fig 3A-D**). Moreover, FTH expansion at d3 was higher in non-colonized participants compared to both low- and high-density colonized participants (**Suppl. Fig 3E**). No consistent changes were observed in maturation/activation of Th subsets.

Thus, although we observed consistent changes in the Th cell compartment at d1 after bacterial challenge, the most prominent change was the expansion of circulating FTH cells at d3 post-challenge. This expansion was only observed in non-colonized participants and not polarized to any subset.

Increased plasma cell numbers at d3 and d11-14 post-challenge

T-cell help, given by FTH cells in the secondary lymphoid organs, is required to activate B cells after antigen encounter.[20] Activation of B cells leads to the formation of memory B cells and plasma cells, and consequently to the production of (protective) antibodies. After (booster) vaccination with aP, skewing towards IgG1 plasma cells was reported, whereas natural encounter is thought to induce IgA memory, as shown by the positive correlation between age and IgA responses against Bp.[21-24]

Upon challenge, fluctuations in the naive and memory B-cell compartment were limited and not consistent in-between participants (**Fig 4A**). However, plasma cells expanded at d11-14 post-challenge and were significantly increased at d14 post-challenge (ratio of baseline) (**Fig 4B, Suppl. Fig 4A**). In participants inoculated with 10^5 CFU, a trend towards higher plasma cell expansion was observed at d11-14 post-challenge (**Suppl. Fig 4B**, ns). At d14 post-challenge, IgG2, IgA1 and IgA2 plasma cells were significantly increased compared to baseline, and for IgG1 this was observed as a trend (p= 0.121) (**Fig 4C-I**). Finally, despite clear expansion, no consistent changes were observed in the distribution of maturation stages of total plasma cells, which were primarily defined by the loss of CD20 and gain of CD138 markers (**Suppl. Fig 4D**).

Next, we grouped the participants based on colonization status. Here, we detected an expansion of plasma cells at d3, which was most prominent in non-colonized participants, but found in seven participants in total (**Fig 5A**, ns). Among all plasma cell subsets, expansion at d3 seemed slightly more prominent for IgG1

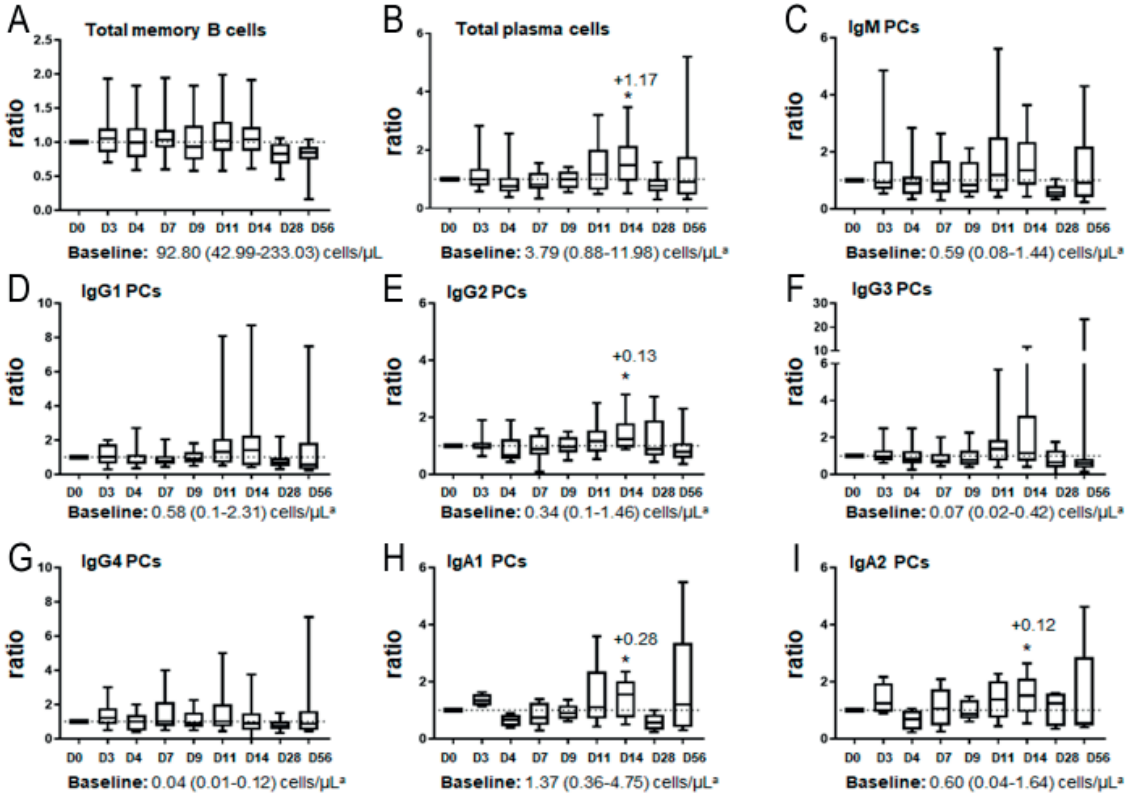


Figure 4. Kinetics in the plasma cell compartment upon bacterial challenge. A. Boxplot (median, Q1, Q3, min-max) showing the expansion of total memory B cells post-challenge. N=15. B-I. Boxplots (median, Q1, Q3, min-max) showing the expansion of total (B), IgM (C), IgG1-4 (D-G), and IgA1-2 (H-I) plasma cells post-challenge expressed as ratio compared to baseline (B-I: N=14). Dashed line indicates a ratio of 1.0 (baseline value). Shown are medians and range. Dashed line indicates a ratio of 1.0 (baseline value). Baseline cell counts, median (min-max) in cells/µl are indicated below each graph. Longitudinal changes were evaluated using Wilcoxon test on ratio of baseline. In case of significant differences in ratio compared to baseline, the median increase in cells/µl is indicated on top of the bar. * Indicates $p<0.05$. PC = plasma cell, d= days post-challenge. a Participant ID.12 showed strongly elevated (IgM) plasma cell counts at baseline, which were about 5x (total plasma cells) and 25x (IgM plasma cells) higher compared to counts measured at day 28 and 56. Therefore, this participant was excluded in panel B-I.

(ns), IgG4 (ns) and IgA1 (ns). Plasma cell expansion at d11-14 seemed unrelated to colonization status (**Fig 5B**). Next, we investigated the maturation patterns of IgG1 and IgA1 plasma cells in colonized and non-colonized participants (**Fig. 5C+D**). We observed that in non-colonized participants the expansion of IgG1 at d3 post-challenge mostly comprised the least and most mature IgG1 plasma cells, whereas for IgA1 this expansion comprised the least and intermediate mature IgA1 plasma cells. For IgG4 plasma cells, cell counts were generally too low to reliably monitor all three plasma cell maturation stages. When assessing the distribution of total plasma cells over different maturation stages, we found a relative increase in more immature plasma cells at d3 and d4 post-challenge in non-colonized participants, but not in colonized participants (**Fig 5C**).

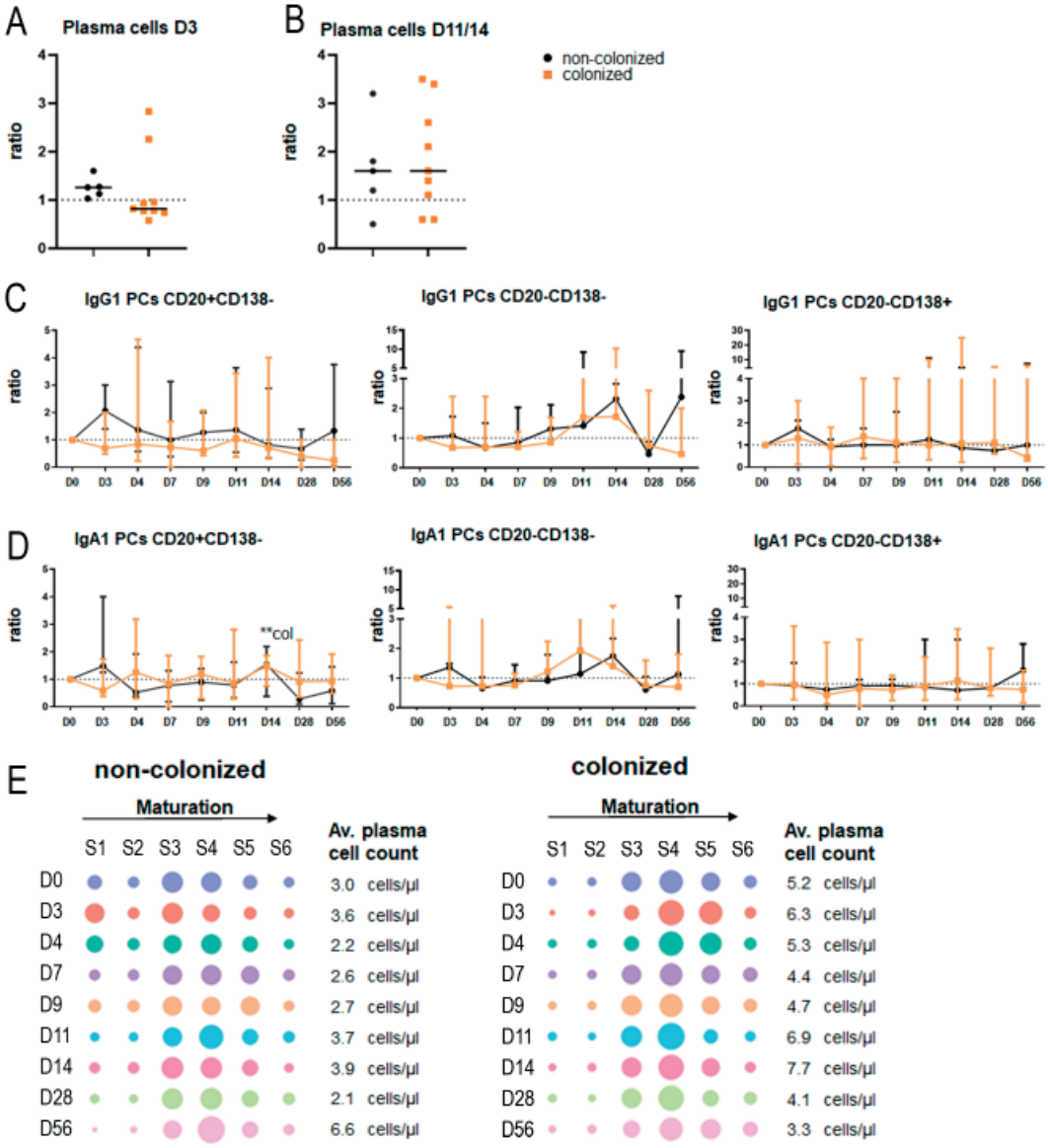


Figure 5. Different kinetics in the plasma cell compartment colonized and non-colonized participants. Of note: due to different normalization of plasma cells in participant ID.12, this participant was not included in the analysis of total plasma cells (panel A, B and E; N=14) A. Plasma cell expansion at d3 post-challenge, expressed as ratio compared to baseline. B. Plasma cell expansion d11-14 post-challenge expressed as ratio compared to baseline (of d11 and d14, the day of maximum expansion was used for each participant). N=14 C+D. Longitudinal changes in IgG1 (C) and IgA1 (D) plasma cell maturation stages expressed as ratio compared to baseline. N=15. Dashed line indicates a ratio of 1.0 (baseline value). E. Distribution of total plasma cells over six different maturation stages (MS1-6), expressed as percentage of total plasma cell population. Average plasma cell counts are indicated at the right side of each plot. N=15 Statistical test performed for longitudinal analysis, Wilcoxon test on ratio of baseline. Statistical test for comparison between groups per time point; Mann-Whitney on ratio of baseline. ** Indicates $p<0.01$.

Then, we divided participants based on colonization density. Aside from non-colonized participants, the plasma cell expansion at d3 was observed in 1 low- and 1 high-density colonized participant (**Suppl. Fig 5A**). Expansion of plasma cells at d11-14 post-challenge was not specific for colonization density (**Suppl. Fig 5B**).

Upon inspection of IgG1 and IgA1 plasma cells, we found that at d3 post-challenge, non-colonized participants had significantly higher numbers of least mature IgA1 plasma cells, as compared to low-density colonized participants. Moreover, both high-density and non-colonized participants showed an increase in intermediate mature IgG1 and IgA1 plasma cells at d11-14 post-challenge (**Suppl. Fig 5C+D**, ns). When assessing the distribution of total plasma cells over different maturation stages, the differences between low-density colonized and non-colonized participants were most prominent at early time points (d0, d3 and d4) (**Suppl. Fig 5E**).

As the expansion of plasma cells at d3 post-challenge seemed more prominent in non-colonized participants, we correlated the plasma cell expansion at d3 with the maximum CFU load and the CFU load at each day post-challenge until the first day of azithromycin treatment. No correlation was found between the plasma cell expansion and the maximum CFU load. Instead, we found a negative correlation between plasma cell expansion at d3 and CFU load at d7 (Spearman R = -0.5773, p=0.0269) and CFU load at d9 (Spearman R = -0.6215, p= 0.0157). Plasma cell expansion at d3 did not correlate with CFU load at d11 or d14. Finally, as T-cell help is required for B-cell activation, we correlated the plasma cell expansion at d3 with Th-cell expansion at d1 and FTH expansion at d3, but no correlation was observed.

Thus, within the B-cell compartment, we observed most changes within the IgG and IgA plasma cell subsets. An early expansion of plasma cells at d3 post-challenge was primarily observed in non-colonized participants, whereas plasma cells at d11-14 post-challenge expanded irrespective of colonization status. A negative correlation was observed between CFU load and plasma cell expansion at d7 and d9 post-challenge. Lastly, maturation of the IgG1 and IgA1 plasma cells was observed to some extent, especially towards to intermediate mature phenotype at d11-14 post-challenge.

Plasma cell expansion and maturation are predictors of seroconversion

As Ag-specific serum Ig levels are routinely used as a readout for vaccine efficacy and/or protective immunity, we also investigated cellular changes associated with seroconversion. Here, we found that all participants who seroconverted (1 low-density and 5 high-density colonized participants) had a more prominent expansion of total plasma cells at d14 post- challenge compared to non-seroconverting participants ($p<0.05$) (**Fig 6A**). This expansion was clearest in IgM, IgG1 and IgA1 plasma cells ($p<0.05$). In participants who seroconverted, the number of intermediate (d14) and most mature (d11 and d14) IgG1 plasma cells was significantly higher compared to non-seroconverting participants (**Fig 6B**). For IgA1 plasma cells, slight differences between seroconverting and non-seroconverting participants were found for the least mature plasma cells (d4 and d9), and a trend towards higher intermediate mature plasma cells was found in seroconverting participants at d11 and d14 (**Fig 6C**). When observing the distribution in matu-

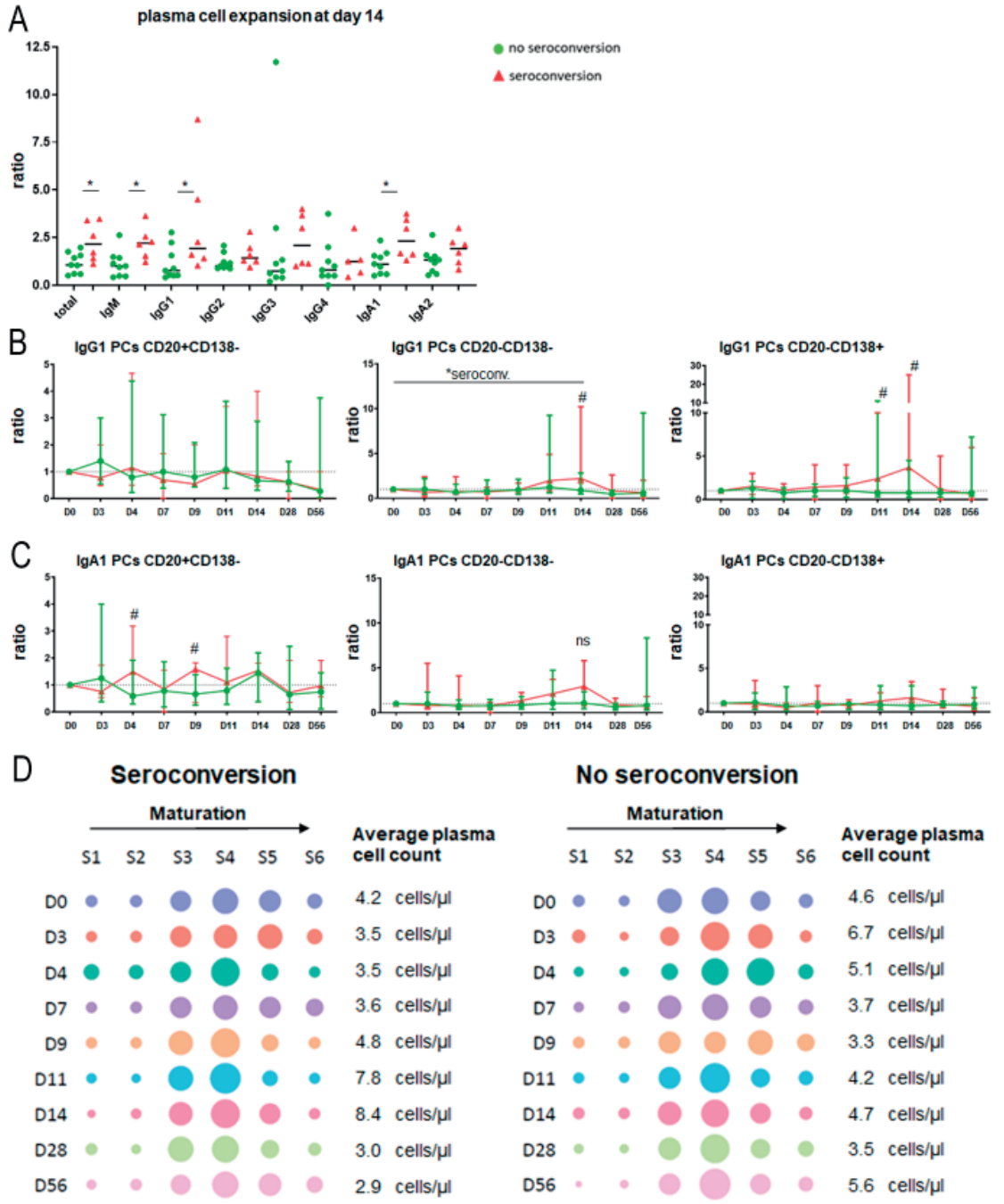


Figure 6. Different plasma cell kinetics in participants that did or did not seroconvert. A. Expansion of plasma cell numbers at d14 post-challenge. Expansion is expressed as ratio compared to baseline. N=15 B+ C. Longitudinal changes in IgG1 (B) and IgA1 (C) plasma cell maturation stages expressed as ratio compared to baseline. N=15. Dashed line indicates a ratio of 1.0 (baseline value). D. Distribution of total plasma cells over six different maturation stages (MS1-6), expressed as percentage of total plasma cell population. Average plasma cell counts are indicated at the right side of each plot. N=15 Statistical test performed for longitudinal analysis, Wilcoxon test on ratio of baseline. Statistical test for comparison between groups per time point; Mann-Whitney on ratio of baseline. * Indicates $p<0.05$ (longitudinal change), # indicates $p<0.05$ (difference between cohorts).

ration stages in the total plasma cell compartment, we again observed the increase in intermediate mature plasma cells in seroconverting participants at d11-d14 post-challenge (**Fig 6D**). In non-seroconverting participants, a slight increase in more mature plasma cells was observed at d3-4 post-challenge.

Evaluation of other immune cell subsets did not reveal any additional cellular changes specific for seroconversion. Lastly, we investigated the correlation between maximum colonization density in CFU during the study and the absolute increase in serum IgG (d28-d0) directed against PT, FHA, Prn and Fim2/3. We found a positive correlation for anti-PT IgG (Spearman R= 0.6486, p=0.0109) and anti-FHA (Spearman R=0.5684, p=0.0296), but not for anti-Prn or anti-Fim2/3 IgG.

Baseline Bp-specific serum IgG levels correlate with maximum CFU count

It is known that baseline status can impact the immune response. Therefore, we correlated the number of plasma cells at baseline and the Bp-specific IgG serum levels with the maximum CFU load. As anti-PT IgG serum levels >20 IU/mL were used as exclusion criteria, we did not correlate baseline anti-PT IgG with maximum CFU count. Still, we found a negative correlation between Bp-specific IgG levels and maximum CFU counts for anti-FHA (Spearman R = -0.5292, p= 0.0447), anti-Prn (Spearman R = -0.6170, p= 0.0166) and anti-Fim2/3 (Spearman R= -0.5210, p = 0.0487), possibly indicating pre-existing immunity in several participants. No correlations were found between baseline plasma cell numbers and the maximum CFU load.

Most informative time points and cell populations associated with protection against colonization.

The here presented study identified the immune cell populations and sampling time points that are most informative for follow-up studies. Especially in the early days after bacterial challenge (d1, d3 and d4), changes in the innate immune compartment are detected. Changes in the adaptive immune response can be monitored at various time points during the two weeks after challenge, with d1, d3, d11 and d14 being most informative in our hands. The most informative time points and cell populations to follow up on after bacterial challenge are summarized in **Figure 7A** (general kinetics post-challenge) and **7B** (kinetics specific for non-colonized participants).

Discussion

This study describes cellular immune responses following bacterial challenge and evaluates which features of this response correlate with protection against colonization and/or seroconversion status. First, we evaluated the general immune cell kinetics post-challenge. Next, we assessed differences in immune signature based on colonization status, density, or seroconversion status. We found that in the early days (d1, 3, 4) after bacterial challenge, multiple cellular changes can be observed in the blood, especially in the neutrophils, monocyte compartments,

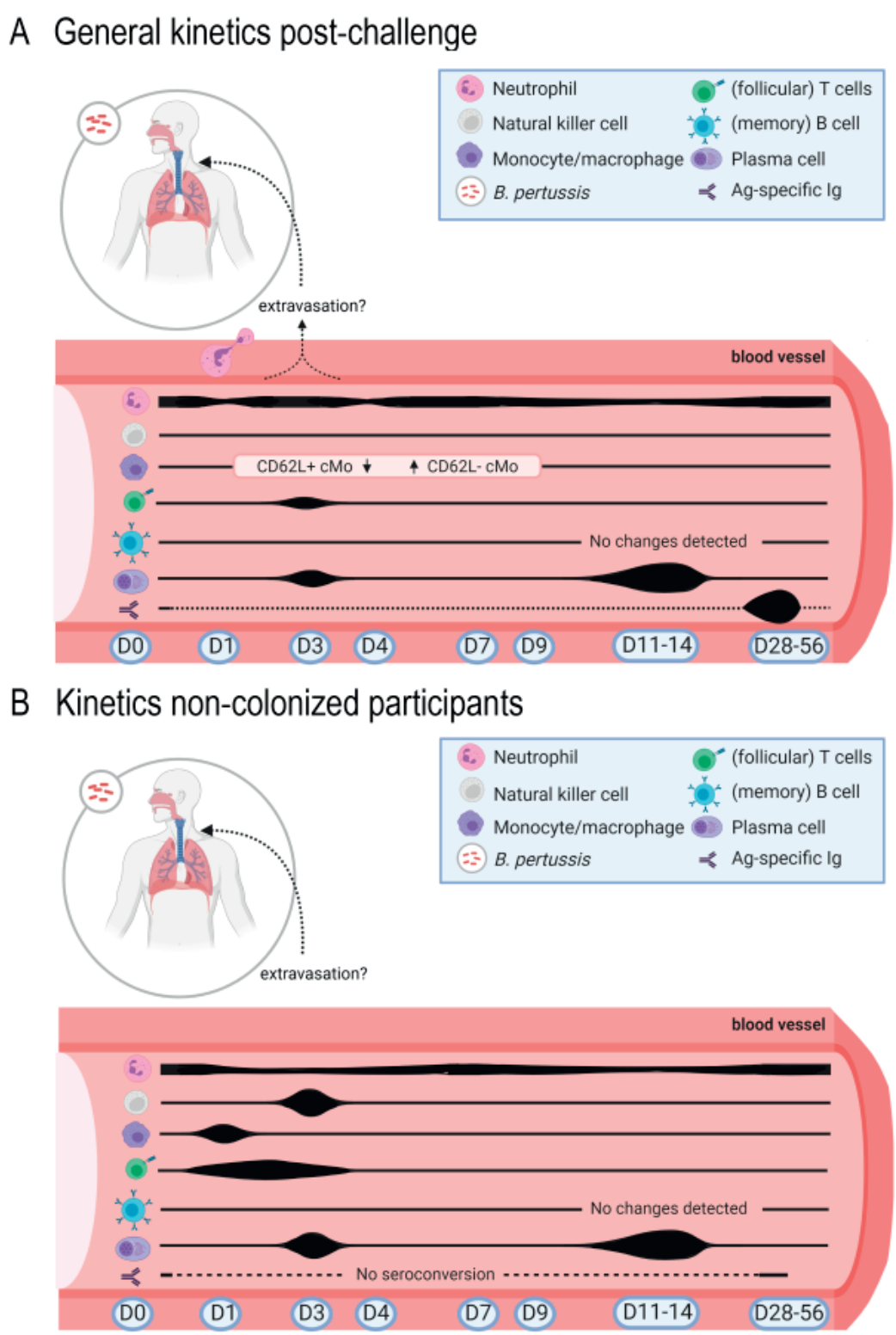


Figure 7. Overview of most informative time points and cell populations after bacterial challenge. A. General cellular kinetics post-challenge irrespective of clinical readout. B. Cellular kinetics unique to participants protected against colonization, possibly indicating pre-existing immunity in these participants.

FTH cells and plasma cells. Kinetics of these subsets differed between colonized and non-colonized participants, and surprisingly, the most prominent differences were found in the response between low-density colonized and non-colonized participants. Interestingly, the d3 plasma cell expansion negatively correlated with CFU load at d7 and d9 post-challenge. Later, at d11-14 post-challenge, expansion of various plasma cell subsets was observed, and increased plasma cell maturation was found in participants who showed seroconversion. Although this exploratory study comprised of only 15 participants, which were clustered based on their colonization status (leading to low statistical power), we detected consistent kinetics related to clinical readouts (colonization and seroconversion status). An important aspect in vaccine evaluation and improvement is understanding the underlying immune response. Cellular immunity against Bp colonization is never studied before in human, but comparisons can be made with vaccination studies, where protection against pertussis is achieved through intramuscular vaccination. Indeed, there are multiple studies describing antibody responses, and some studies also describe cellular responses induced by intramuscular vaccination against pertussis.[17,21-23,25] The major differences between current Bp vaccines and natural infection are the location of antigen encounter (intramuscular vs. mucosal) and the variety and concentration of antigens (aP vaccines contain purified Bp antigens usually combined with other bacterial/viral compounds like tetanus, diphtheria, polio). Although Bp vaccines confer protection against disease, they do not seem to prevent colonization and transmission of the bacterium.[3,26,27] Therefore, it is of critical importance to understand what is required to prevent colonization, translocation, and transmission of Bp. The benefit of reduced carriage has already been demonstrated for meningococcal disease and *Haemophilus influenzae* type b, where reduced carriage resulted in lowered transmission and increased herd immunity. This is most likely related to better mucosal immunity.[28-30]

In two previous vaccination studies, we explored the cellular responses to intramuscular aP booster vaccination in various cohorts using highly similar methods (aP-primed children, aP- or wP-primed adolescents, wP-primed young adults, and wP-primed older adults).[21,31] Both studies pointed towards the d7 plasma cell expansion and maturation as the major cellular change post-vaccination. One of these studies also investigated the innate and T-cell kinetics in 10 wP-primed participants who received an aP booster.[21] There, we observed 'late' monocyte kinetics at d3 post-vaccination (mostly iMo and ncMo) in approximately half of the participants. No consistent changes were observed post-vaccination in the numbers or composition of the CD4 T-cell, CD8 T-cell, or NK cell compartment. Comparing these studies with the here presented controlled infection study, we found clear differences between the kinetics post-challenge and post-vaccination, which are discussed in more detail below. Interestingly, several of the findings in this challenge model were in line with in a recent study evaluating the immune responses after intranasal vaccination with an attenuated Bp strain.[10]

Of all evaluated innate immune cells, especially monocyte subsets hold promise

for future evaluation. The decrease of CD62L+ cMo and increase of CD62L- cMo implies activation and maturation of the cMo. This activation was seen in all challenged participants irrespective of clinical readout. However, the early expansion of (especially) CD36- ncMo was found exclusively in participants protected against colonization. Currently, information about the exact role of CD36- ncMo is scarce. However, part of the CD36- ncMos are SLAN+ ncMos. SLAN+ ncMos were recently reviewed by Ahmad et al [32] and are known to be circulating and tissue myeloid cells with high plasticity and a pro-inflammatory function. *In vitro* studies showed that SLAN+ ncMos could rapidly differentiate into macrophages [13,33,34] or acquire DC functions, [35,36] and had a greater capacity to prime naive T cells towards Th1/Th17 cells when compared to CD1c+ DCs.[32,36-38] Although these findings were not obtained in the context of Bp infection, the ability to promote Th1 and Th17 responses is relevant in the case of Bp infection, implying that SLAN+ ncMos may contribute to the (protective) immune response. Consequently, we believe that monocyte subsets should be included in future immune monitoring studies.

In our previously published vaccination study, we did not observe the activation of cMo or the expansion of CD36- ncMo, although an expansion of overall iMo and ncMo was found.[21] This may be explained by the lack of early time points after vaccination or by the different mode of antigen encounter. Recent work on intranasal vaccination with a life attenuated Bp (BPZE1) did not observe consistent changes or maturation in cMo, iMo or ncMo either.[10] This may be attributed to differences in methodology, such as depth of evaluation, (number of) evaluated time points or the use of an attenuated Bp strain. Interestingly, the authors reported the production of several proinflammatory cytokines by purified monocytes upon stimulation with BPZE1, implying that upon contact with Bp, the monocytes start a local inflammation response.

We observed a decrease in circulating mature neutrophils several days post-challenge, which was a bit more prominent in the participants protected against colonization. The decrease in mature neutrophils may imply that these cells have been recruited into the tissue. Recent mouse studies indicated a role for IL-17, secreted by tissue-resident CD4 T cells (Trm cells), which is expected to play a role in the recruitment of neutrophils and enhancing Bp clearance.[39,40] Of note, those studies primarily focused on neutrophils present in (nasal) tissue, which may not be easily integrated in human studies due to the more invasive nature of sampling of tissue-resident cells (e.g. via nasal scrapings). As the decreased number of circulating mature neutrophils observed in this study may imply neutrophil extravasation, this (mature) population should be considered in future investigations.

The expansion of NK cells seemed to have an inverse relation with colonization density. This expansion was not observed upon booster vaccination with aP, neither did the intranasal study report on NK cell kinetics.[10,21] Animal studies have shown a role for NK cells in Bp infection, where they are thought to be the

initial source of IFNγ (reviewed by Higgs et al., [41]). The production of IFNγ is essential for containment of the infection and promotion of the Th1 response. Although we did not monitor IFNγ secretion, we did find an early specific NK-cell signature (expansion) in the non-colonized participants.

Interestingly, we found that low-density colonized donors showed more prominent innate immune cell kinetics compared to high-density colonized participants. A possible explanation might be the presence of some pre-existing immunological memory, yet not as high as in participants protected from colonization. This pre-existing memory might result in a more targeted innate immune response (influenced by pre-existing memory cells or antibodies) and faster control of bacterial growth as compared to donors without any noteworthy pre-existing immunological memory.

We found an increase in FTH cells at d3 post-challenge, which was especially prominent in non-colonized participants. Although we did not find such expansion after aP booster vaccination, the flow cytometric analysis of FTH cells after intranasal pertussis vaccination (with BPZE1) did report an expansion of activated (as measured by expression of PD-1 and ICOS) Th1-like FTH cells starting at d4 post-vaccination.[10] We did not assess activation status of the FTH cells, but did observe an expansion of FTH cells at d3 post-challenge. Activation and/or expansion of FTH cells can support formation of germinal centers, and thus initiate humoral responses, leading to the generation of (protective) antibodies. Moreover, we found increased numbers of circulating T-helper cells of the Th1/17, TH22 and CXCR3-CCR4-CCR6+CCR10- phenotype at d1 and d3 post-challenge. These changes were not observed after an aP booster vaccination, and are much earlier than the T-cell readout that was used in the evaluation of BPZE1 vaccination, where a Th1-response was found in stimulated PBMCs at d28 post-vaccination.10 In this study, we observed expansion of several IgG and IgA plasma cell subsets at d3 (mostly in non-colonized participants) and at d11-14 post-challenge. This is different compared to post-vaccination kinetics, where we observed a prominent and homogeneous expansion and maturation of (primarily IgG1) plasma cells at d7 post-vaccination. This difference may be explained by the way that the antigen is administered. During the booster vaccination, a high dose of antigen (+ adjuvant) is injected into the muscle, and thus all participants will be exposed to the antigen at the same time (d0), resulting in a synchronized induction of the immune response in vaccinated donors. Upon intranasal bacterial challenge, all participants are challenged with Bp at d0, but there may be differences in bacterial growth between participants. Therefore, the plasma cell peak may differ in timing between participants and thus not be as homogenous as after receiving a booster vaccination. Like in the post-challenge data, the expansion of plasma blasts (total, no subsets identified) after intranasal vaccination peaked at d14.[10] Interestingly, in that same set of data, in 2/12 participants an expansion of plasma blasts/plasma cells seemed present already at d4 post-vaccination, although the authors did not comment on this.

The observed IgG response is most likely the result of previous vaccine-induced immunity against Bp and natural boosting, whereas the IgA response can be mainly attributed to previous natural infections. It should be noted that once generated, both IgG and IgA memory B cells may be triggered by vaccination and/or infection. As natural infection occurs via the respiratory tract, local mucosal immunity (i.e., secretory IgA antibodies) is of importance in the rapid removal/neutralization of Bp upon infection. Increased mucosal immunity should be an important aspect for future Bp vaccine candidates, as this may reduce transmission. However, we believe that before this can be evaluated, the cellular responses preventing colonization and transmission should be understood. Of course, the question is to what extent the circulating IgA plasma cells represent mucosal IgA responses. Additional analysis of surface or intracellular markers may give insight in the destination of these plasma cells, for example, the use of markers that allow to differentiate between circulating and mucosa-oriented plasma cells (such as adhesion molecule β7 integrin, J-chain or C-C motif receptor 10 (CCR10) [42-44]), may help to understand the destination of the expanding plasma cells. Expression of the J-chain would be of special interest for the peak of plasma cells that was observed at d3 post-challenge in the participants who were protected from colonization, as expression of mucosal homing markers may be lost when these cells are recruited from mucosal layers into the circulation. Moreover, repertoire studies comparing the (Ag-specific) B-cell receptor (BCR) repertoire between B cells at baseline, the plasma cells at d3 and at d11-14, and memory B cells at later time points may yield valuable insights in the relationship of these cells. Such repertoire studies could give insights in the breadth of the antibody response between challenged and vaccinated individuals. Additionally, (Ag-specific) BCR repertoire studies may also identify differences in antibody reactivity between high-density, low-density, and non-colonized participants, possibly showing selection towards a shared (protective) response to specific antigens/epitopes in participants protected against colonization. Lastly, it may be of interest to determine the clonal relationship between the plasma cells at d3 and the plasma cells that arise at d11/14, to see whether these d3 plasma cells are indeed derived from pre-existing immunity.

In this study, all participants were inoculated with predefined doses of a well-characterized Bp strain. We acknowledge that a controlled experimental infection is not the same as a natural infection model. The mode of infection, the dose and circumstances are designed to be standardized and this might be a limitation to translate these findings to natural pertussis infection. Nevertheless, we clearly observed shared kinetics within the total cohort, and when stratifying based on colonization status.

Not all participants who were colonized, showed signs of seroconversion. Perhaps translocation of Bp is required to initiate seroconversion. In that case, although these participants were colonized, the bacteria did not fully translocate into the tissue in all participants, leading to seroconversion only in the participants where bacteria had translocated. It may be of interest to compare the kine-

tics of antibodies in the mucosal lining fluid of the nose in participants who did and did not seroconvert and their colonization density. Additionally, it may be of interest to study whether the mucosal antibodies and the early plasma cell peak are associated with each other (e.g. by comparing BCRs). Moreover, it may be interesting to investigate whether participants can transmit Bp to others, as this would confirm that asymptomatic infection results in transmission of Bp. Such finding would imply that the presence/absence of symptoms and the serology status may not correctly reflect carriage/transmission state, and thus that serological surveillance studies, although valuable, may still underestimate the number of Bp infections.

In this cellular immune monitoring study, we evaluated the overall immune cell kinetics. We previously showed that there is a fair correlation between Ag-specific ELISpot data and plasma cell flow cytometry data in post-vaccination settings [45]. Although no ELISpot experiments were performed in the here-reported study, we did observe an increased number of (total and more mature) plasma cells at d14 post-challenge in donors showing seroconversion. The latter is in line with the ELISpot data that was previously reported on the total cohort used in the dose-finding study of this human challenge model.[12] There, increased numbers of Ag-specific Ig producing plasma cells were observed in multiple colonized participants (n=16) at d14 post-challenge, but not in participants protected from colonization (n=9). In both studies seroconversion was only observed in colonized participants. Given the relationship between Ag-specific Ig producing plasma cells and the serum Ig levels, the participants showing increased number of Ag-specific Ig producing plasma cells are likely the donors with seroconversion. Aside from ELISpot, an Ag-specific approach combined with mucosal-tracing markers may be informative, possibly helping us to find (surrogate) biomarkers of protection in the blood. An antigen-specific approach would be especially relevant to monitor kinetics of specific memory B- and T- cells.

One remaining question is whether the early response observed in participants protected from colonization is indeed protective, and to what extent this information can be used in future studies. To confirm whether a protective response is observed in participants, in fact a rechallenge would be required (e.g., an infection-reinfection cohort). As we know that natural infection gives the best protection (compared to wP and aP vaccination) [3,6], it may be that this controlled infection leads to a similar level of protection. Based on our data, we hypothesize that the initial decrease in mature neutrophils, and early expansion of CD36- ncMos, NK cells, FTH T cells and plasma cell in the non-colonized participants may be associated with an efficient mucosal immune response, preventing colonization. When re-challenging the same group of individuals, we would expect a higher number of participants to be protected against colonization, and thus to show this same early cellular response.

To the extent of our knowledge, we are one of the first to investigate such in-depth cellular immune responses in a Bp human challenge model. Here, we report which

cell populations and time points can yield valuable information about the colonization status and how this compares to vaccine-induced cellular kinetics. Because of the low incidence of pertussis and the difficulty to diagnose it early, there is a lack of information on cellular responses upon Bp infection in humans. Increased insight, especially in the protective immune signature, is crucial to the development of novel pertussis vaccines, which should aim to prevent colonization and transmission of Bp. This study increases insights in the cellular responses in individuals with varying degree of colonization upon controlled bacterial challenge. As we only investigated 15 participants, these findings should be corroborated in future studies. When fully established, this human challenge model can not only be used to study Bp infection, colonization, translocation, transmission and/or shedding in challenged individuals, but also to dissect the induced immune responses: early vs late responses, mucosal vs systemic responses, and protective vs non-protective responses. More importantly, controlled bacterial challenge could be an important step to evaluate the efficacy of novel Bp vaccine candidates with respect to protection against colonization and transmission.

Experimental methods

Inclusion criteria and sample collection

Peripheral blood (PB) samples were collected during a dose-finding controlled infection study using Bp (NCT03751514, ethical committee reference 17/SC/0006), which aimed to determine the dose required to colonize at least 70% of participants, and was reported by De Graaf et al. [11,12] Participants (wP-primed) were eligible for the study when they were healthy, aged 18-45 years, available for the 16-day admission period and scheduled visits, vaccinated against Bp at least 5 years before enrollment, did not use antibiotics within 4 weeks before enrollment of the study, had no contraindication to azithromycin (administered at d14, d15, and d16) and had no contact with individuals vulnerable to pertussis (full eligibility criteria were reported before [12]). Participants with recent exposure to Bp, defined as baseline serum anti-PT IgG of >20 IU/mL (determined by ELISA), were excluded. Participants were challenged and admitted to the National Health Institute for Health Research Clinical Research Facility in Southampton, UK. A total of 34 participants were challenged with various doses of Bp. This paper describes 15 healthy participants who were challenged intra-nasally with 10^4 (n=5) or 10^5 (n=10) CFU of Bp strain B1917 (n=15, m/f ratio: 8/7, age range: 18-43 years old).

Colonization was assessed by culture of nasal wash samples on Bp-specific media at d4, d5, d7, d9, d11, d14, d15 and d16 post-challenge.[12] Colonization was defined as any Bp-positive culture at any time point post-challenge. Colonization density was stratified into 3 categories: non-colonized (0 CFU/mL), low-density colonized (< 1000 CFU/mL) and high-density colonized (> 1000 CFU/mL) at any time point.

PB was collected in EDTA blood collection tubes at d0, d1, d3, d4, d7, d9, d11, d14, d28 and d56 post-challenge. PB was taken early morning and transported

to Leiden University Medical Center (LUMC), the Netherlands, where flow cytometric evaluation was performed within 12h after donation. In two participants (ID.08 and ID.13), the expression of CD45RA on lymphocytes was found to be unusual, most likely caused by a CD45RA-related polymorphism19, hampering reliable identification of most T-cell subsets in these two participants. Aside from the anti-PT ELISA used for initial screening, serum IgG against PT, Prn, FHA and Fim2/3 was measured at baseline and d28 using multiplex immune assay (MIA) [46] at the National Institute for Public Health and the Environment (RIVM, The Netherlands). in this study, seroconversion is defined as an >2-fold increase in anti-PT IgG compared to baseline.

Blood processing and staining – immune monitoring panels and absolute count determination

All PB samples were subjected to high-throughput EuroFlow-based flow cytometric immunophenotyping with four multicolor immune monitoring panels (or their direct prototypes). These panels allow monitoring the kinetics of over 250 circulating immune cell subsets. In short, the B-cell and plasma cell tube (BIGH) allows identification of up to 115 B- and plasma cell subsets, that are distinguished based on expressed Ig subclasses and their maturation stage-associated phenotype.[45,47] The CD4 T-cell panel (CD4T) allows identification of >89 CD4 T-cell subsets with different functionalities and maturation stages.[18] The CD8 cytotoxic T-cell panel (CYTOX) allows identification of up to 50 subsets within the CD8 T-cell and natural killer (NK) cell compartment.[47] Lastly, the dendritic cell-monocyte panel (DC-Monocyte) allows identification of up to 19 different (sub)populations within the myeloid compartment, including subsets of monocytes and dendritic cells [van der Pan et al., manuscript submitted].

All blood samples were processed according to the EuroFlow standard operating protocol (SOP) as reported previously (protocols available at www.EuroFlow.org).[21,45] Two adjustments of the previously reported methods concerned the addition of surface marker CD45 to the BIGH, CD4T and CYTOX panel, and the cytoplasmic staining with a CD154 antibody in the CD4T and the CYTOX panel. The latter resulted in an additional intracellular staining step for the CYTOX panel.

In short, for CD4T and CYTOX panel 100µL PB was used for a membrane and intracellular staining using the Fix and Perm kit (Sanbio). For the BIGH and DC-Monocyte panel, high cell numbers were required, and thus a bulk lysis was performed on whole blood, then 10 million cells were stained for membrane markers. For the BIGH panel, this was followed by intracellular staining for immunoglobulins (again, using the Fix and Perm kit). For the DC-Monocyte panel, the surface staining was followed by a 10 min incubation with BD FACS lysing solution (BD Biosciences, San Jose, CA, USA), washed and acquired on the flow cytometer.

Absolute cell counts were determined with the use of Perfect-Count Microsphe-

res™ (Cytognos, Spain) according to the EuroFlow SOP (www.EuroFlow.org). In short, exactly 50µL of PB was stained with antibodies directed against CD19, CD3 and CD45, and samples were incubated for 30 min in the dark. Next, 500µL of NH4CL was added, and after 10 min incubation exactly 50µl of Perfect-Count Microspheres™ were added. Samples were acquired immediately after. This procedure allowed for accurate assessment of absolute cell counts of leukocytes, lymphocytes, B-, T- and NK cells.

All samples were measured on a BD FACS LSR Fortessa 4L or on a BD FACS LSR Fortessa X-20 4L (BD Biosciences, San Jose, CA, USA). Flow cytometer performance was assessed daily according to the EuroFlow guidelines as previously described.[48,49] Staining with all antibody panels was performed at every time point, with exception of d1 post-challenge, at which the BIGH panel was not applied.

Data analysis and statistics

All data were analyzed manually in the Infinicyt™ Software (v2.0, Cytognos, Spain) according to the EuroFlow gating strategies.[18,45,47] [Van der Pan et al, manuscript submitted] Plasma cell maturation stages were defined using the maturation pathway tool in the Infinicyt software, as previously described.[21]

Differences in cell counts between multiple (>2) conditions were assessed with Kruskal-Wallis, followed by Dunn's test. Differences between cell counts/ratios in two tested conditions were assessed with Mann-Whitney test. A p-value <0.05 was considered statistically significant. GraphPad Prism 8.0 software (GraphPad, San Diego, CA, USA) was used for statistical tests. Correlations between two parameters were assessed using Spearman's ranking correlation.

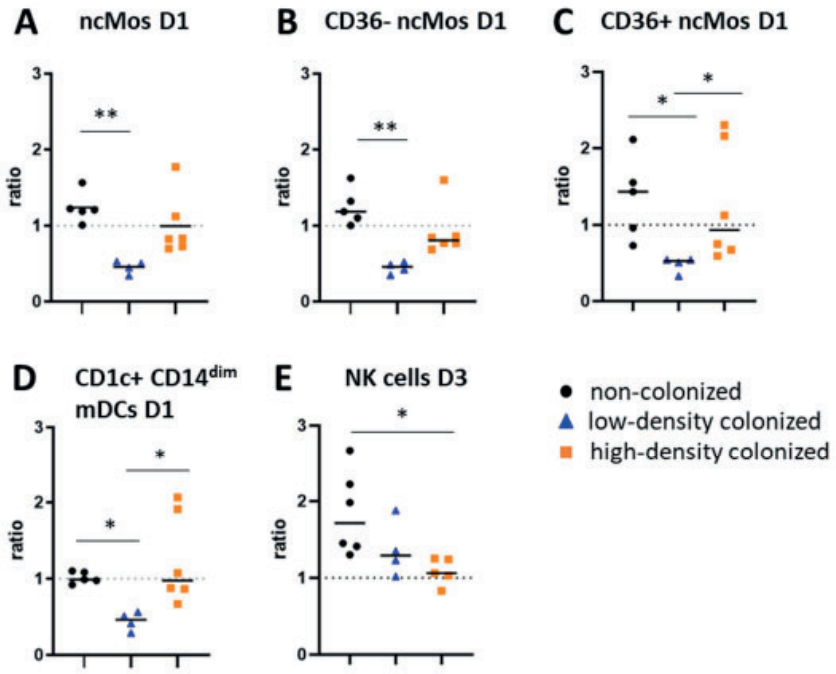
Acknowledgements

The authors would like to thank the nurses for their help in this study, Suzanne Comans for her help with data analysis and all participants for their participation in this study. We thank Indu Khatri for her help in constructing the Bubble Plots in Python. Moreover, we are thankful for Anna De Schutter, Sarah Horswill, Rosy Haig, and FlyBe Airlines for their help in transferring the samples from UK to the Netherlands. The authors gratefully acknowledge the Flow cytometry Core Facility (FCF) of Leiden University Medical Center (LUMC) in Leiden, the Netherlands (https://www.lumc.nl/research/facilities/fcf), coordinated by M. Hameetman, run by the FCF Operators, D.M. Lowie, S. van de Pas, G.IJ. Reyneveld, and former FCF coordinator dr. K Schepers and former operators J.P. Jansen, E.F.E de Haas, G.M. De Roo, R.J. Mclaughlin and S.A.J. Veld (Directors: Prof. F.J.T. Staal and Prof. J.J.M. van Dongen) for their technical support. The data safety and monitoring committee: Dr. Stephen Gordon, Dr. Wei Shen Lim, Dr. Patrick Lillie. The nurses and coordinating staff of the NIHR Clinical Research Facility Southampton (director: Prof. S.N. Faust). The scientific advisory committee: Prof Andrew Gorringe, Dr. Kent Kester and Dr. Dimitri Diavotopoulos.

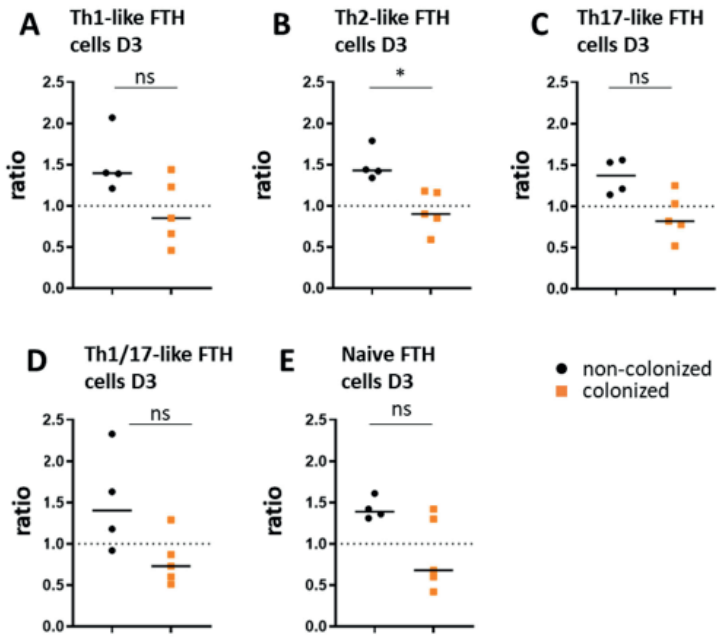
Funding
This PERISCOPE project has received funding from the Innovative Medicines Initiative 2 Joint Undertaking under grant agreement No 115910. This Joint Undertaking receives support from the European Union's Horizon 2020 research and innovation program and EFPIA and BMGF. This manuscript only reflects the author's views. The Joint Undertaking is not responsible for any use that may be made of the information this manuscript contains. The flow cytometric studies were supported by the EuroFlow Consortium, which received support from the FP6-2004-LIFESCIHEALTH-5 program of the European Commission (grant LSHB-CT-2006-018708) as Specific Targeted Research Project (STREP).

Conflict of interest
J.J.M. van Dongen is chairman of the EuroFlow scientific consortium, which was initially supported by the FP6-2004-LIFESCIHEALTH-5 program of the European Commission (grant LSHB-CT-2006-018708) as Specific Targeted Research Project (STREP); now the EuroFlow consortium receives royalties from licensed patents, which income is solely used for continuation of the collaboration within the consortium. In addition, J.J.M. van Dongen has an Educational Service Agreement with BD Biosciences and a Scientific Advisory Agreement with Cytognos; income from these agreements goes to LUMC. Lastly, J.J.M. van Dongen, M.A. Berkowska, C.I. Teodosio and A.M. Diks are listed as (co)inventors on the patent issued by the EuroFlow scientific consortium, which describes the flow cytometry panels used in this study. Other authors declare no conflict of interest.

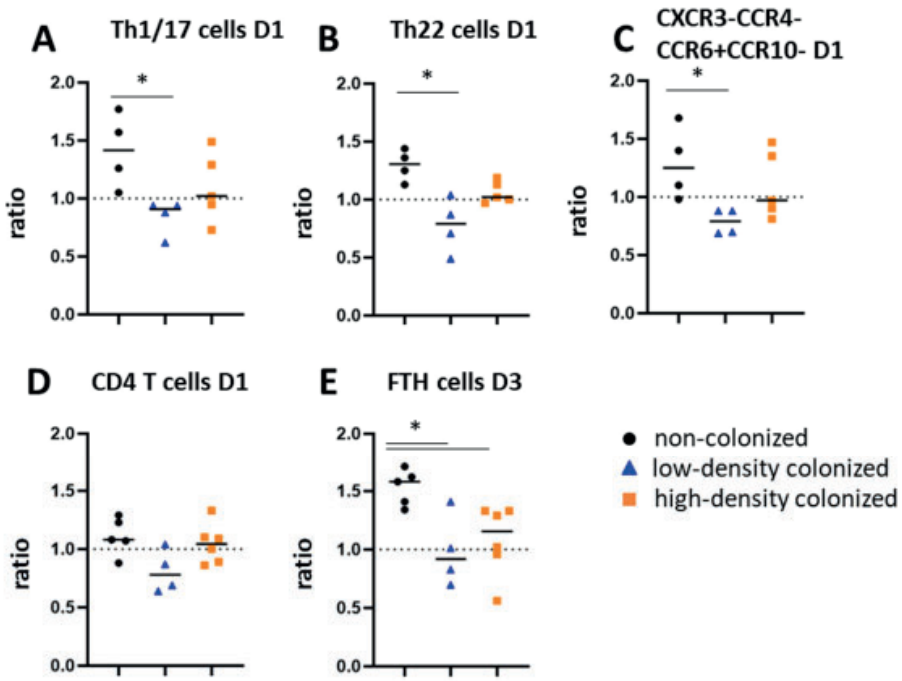
Supplemental Materials
This work is still a manuscript in preparation. Therefore, all supplemental material is shown on the following pages.



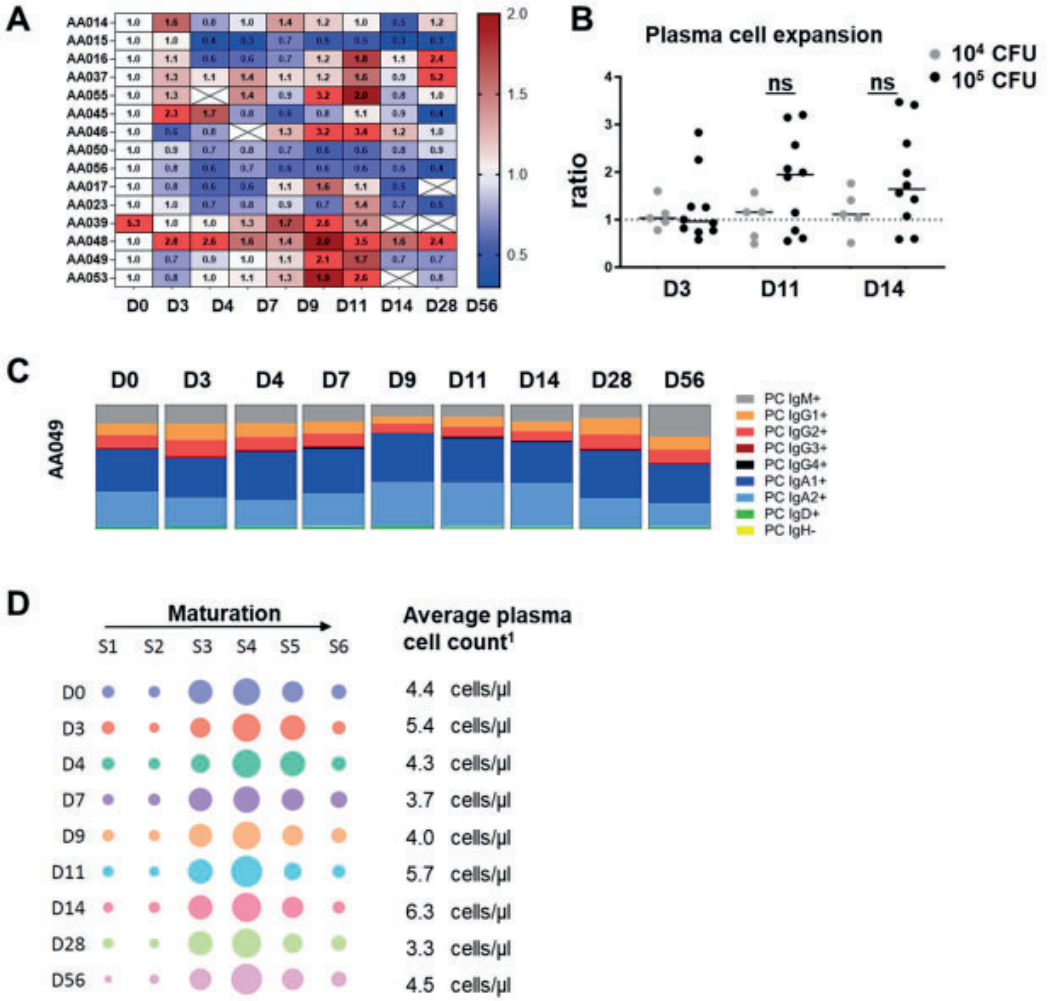
Supplemental Figure 1. Different kinetics in the innate immune cell compartment based on colonization density. Differences are presented as ratio of baseline. Dashed line indicates a ratio of 1.0 (baseline value). Kruskal-Wallis followed by Dunns' test was used to assess differences. D= days after challenge. * p<0.05, ** p<0.01.



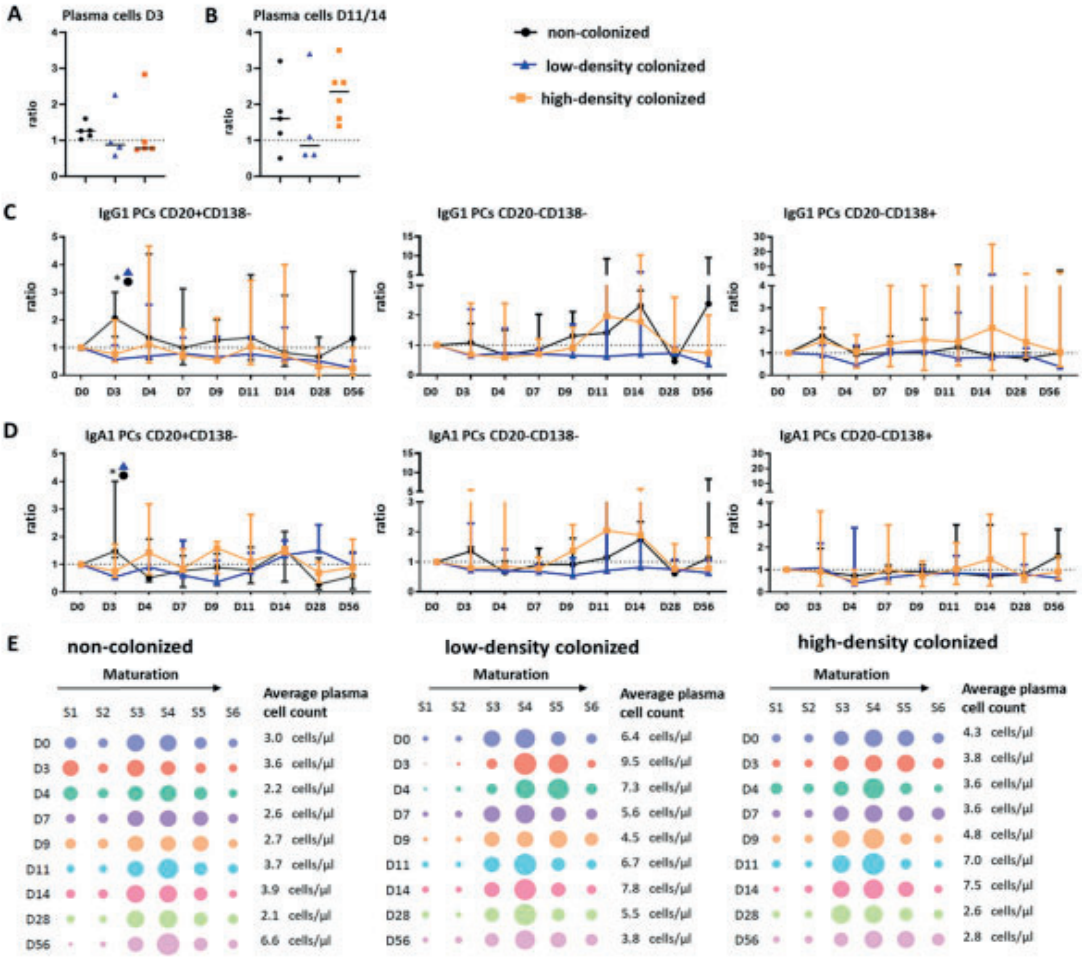
Supplemental Figure 2. Expansion of follicular T helper (FTH) cells in participants protected from colonization. Expansion of FTH subsets at d3 post-challenge expressed as ratio of baseline. Dashed line indicates a ratio of 1.0 (baseline value). Of note, due to technical limitations, in 6/15 participants no FTH subsets could be defined. N=9. Differences between groups were assessed using Mann-Whitney test. * p<0.05. D= days after challenge.



Supplemental Figure 3. Kinetics of T helper cells in participants grouped upon colonization density. Expansion of reduction of circulating cells was expressed as ratio of baseline. Dashed line indicates a ratio of 1.0 (baseline value). Kruskal-Wallis followed by Dunns' test was used to assess differences. * p<0.05. D= days after challenge, FTH= follicular T helper cells, Th cell= T helper cell.



Supplemental Figure 4. Kinetics in the plasma cell compartment upon bacterial challenge. A. Heatmap representing the expansion of plasma cells in ratio compared to baseline ranked based on colonization status. NB: Participant ID.12 showed a strongly elevated number of IgM plasma cells at baseline. Dashed line indicates a ratio of 1.0 (baseline value). Therefore, it was decided to normalize the plasma cell numbers of this participant to d3 instead of baseline. B. Expansion of plasma cell in ratio compared to baseline. The cohort is split based on initial CFU dosage (10^4 or 10^5 CFU of BP1917) received at the day of challenge. Each dot represents one participant, the bar indicates the median value of all participants in each cohort. C. Fluctuations in the distribution of the plasma cell compartment over time. One representative participant is shown. D. Per time point the percentage of plasma cells in each maturation stage was plotted (total plasma cells in donors that that were or were not colonized, grouped per time point). The size of the dot indicates the % of plasma cells in each maturation stage (average of participant). Cell count is shown at the right side of the plot (average of participants). Bubble plots were generated using plotly python graphing library.1 Participant ID.12 was not included in panel D due to deviating baseline IgM plasma cell counts, possibly hinting at an ongoing immune response at time of challenge. D= Days after challenge.



Supplemental Figure 5. Plasma cell kinetics in participants that were non-colonized, low-density colonized, or high-density colonized. A. Plasma cell expansion at d3 post- challenge. Expansion expressed as ratio compared to baseline. Each dot represents one participant, the bar indicates the median value of all donors each cohort. B. Plasma cell expansion at d11/14 post-challenge. Expansion expressed as ratio compared to baseline. For panel A and B, Kruskal-Wallis followed by Dunns' test was used to assess differences. C+D. Total plasma cells were divided into three different maturation stages (CD20+CD138-, CD20-CD138-, CD20-CD138+). Per maturation stage, the ratio compared to baseline was compared between donors of the three groups for IgG1 (C) and IgA1 (D) plasma cells. E. Per time point the percentage of plasma cells in each maturation stage was plotted (total plasma cells in donors that that were or were not colonized, grouped per time point). The size of the dot indicates the % of plasma cells in each maturation stage (average of donors). Cell count is shown at the right side of the plot (average of participants). Bubble plots were generated using plotly python graphing library. Dashed line indicates a ratio of 1.0 (baseline value). Statistical test performed for longitudinal analysis: Wilcoxon. Statistical test for comparison between groups per time point; Mann-Whitney. D= days after challenge.

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