Anaplasma phagocytophilum infects cells of the megakaryocytic lineage through sialylated ligands but fails to alter platelet production

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Anaplasma phagocytophilum is an obligate intracellular bacterial pathogen that principally inhabits neutrophils. However, infection with *A. phagocytophilum* results in a moderate to marked thrombocytopenia. In host neutrophils, *A. phagocytophilum* uses sialylated ligands, primarily P-selectin glycoprotein ligand-1 (PSGL-1), to enter its host cell. PSGL-1 is expressed on a wide array of haematopoietic cells, including megakaryocytes. In this study, it was hypothesized that (i) cells of the megakaryocytic lineage (MEG-01 cells) would be susceptible to *A. phagocytophilum* infection and (ii) infection may induce alterations in platelet production contributing to infection-induced thrombocytopenia. It was found that MEG-01 cells are susceptible to infection. MEG-01 cells expressing abundant sialylated ligands were the most susceptible to infection, and the absence of sialylation, or blocking of PSGL-1, limited infection susceptibility. However, infected MEG-01 cells produced proplatelets and platelet-like particles comparable to uninfected cells. These results highlight a novel target of pathogen infection and suggest that the pathogen may utilize similar strategies to gain access to megakaryocytes. Direct pathogen modification of platelet production may not play a role in infection-induced thrombocytopenia.

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INTRODUCTION

Anaplasma phagocytophilum, an obligate intracellular bacterium, is the causative agent of granulocytic anaplasmosis (GA), formerly known as granulocytic ehrlichiosis (Chen *et al.*, 1994; Dumler *et al.*, 2001). GA is an emerging, zoonotic, tick-borne disease spread by *Ixodes* spp. tick vectors. Regardless of host species, infection with *A. phagocytophilum* results in hallmark haematological alterations, most notably a moderate to marked thrombocytopenia (Bakken *et al.*, 1996, 2001; Borjesson *et al.*, 2001). Thrombocytopenia may be accompanied by a mild to moderate leukopenia and mild anaemia (Bakken *et al.*, 1996; Borjesson *et al.*, 2001; Madigan & Gribble, 1987; Pusterla *et al.*, 1999). The mechanism of thrombocytopenia and other cytopenias is unknown.

A. phagocytophilum primarily infects host neutrophils. However, it is also capable of infecting other haematopoietic and non-haematopoietic cells including human bone marrow-derived CD34⁺ cells (Klein *et al.*, 1997) and endothelial cells (Munderloh *et al.*, 2004). Pathogen infection of human neutrophils has been shown to occur through binding of P-selectin glycoprotein ligand-1 (PSGL-1) and sialylated and α 1,3-fucosylated glycans such as the sialyl Lewis x (sLe^x) antigen (Goodman *et al.*, 1999; Herron *et al.*, 2000). Specifically, sLe^x is important for *A. phagocytophilum* invasion (Goodman *et al.*, 1999) and the PSGL-1 N-terminal peptide is important for both bacterial binding and internalization (Herron *et al.*, 2000). We have recently shown that *A. phagocytophilum* can also adhere to and infect host myelocytic cells in a sialic acid- and PSGL-1-independent manner (Reneer *et al.*, 2006; Sarkar *et al.*, 2007). A variety of haematopoietic cells express PSGL-1, including platelets and their progenitor cells, megakaryocytes (MKs) (Frenette *et al.*, 2000). Combined, these data suggest that other haematopoietic cells, notably MKs, may be

Abbreviations: FBS, fetal bovine serum; GA, granulocytic anaplasmosis; MK, megakaryocyte; p.i., post-infection; PLPs, platelet-like particles; PSGL-1, P-selectin glycoprotein-1; sLe^x, sialyl Lewis x.

susceptible to *A. phagocytophilum* infection via bacterial interactions with sLe^x and PSGL-1. Infection of haematopoietic precursors may contribute to infection-induced cytopenias.

Mammalian platelets are anucleate cells, derived from MKs, that carry out little *de novo* protein synthesis. Platelets are produced by mature, differentiated MKs in the bone marrow where long, cytoplasmic processes (proplatelets) are extended into marrow sinusoids (Italiano *et al.*, 1999). MK cell lines are often employed for studies on platelet production. The leukaemic megakaryoblastic cell line MEG-01 has been shown to produce proplatelets and platelet-like particles (PLPs) in cell culture (Takeuchi *et al.*, 1998). Additionally, these cells can be induced towards differentiation and maturation using various agents including phorbol-12-myristate-13-acetate, an anti-Fas antibody (CH11) and thrombopoietin (Battinelli *et al.*, 2001; Clarke *et al.*, 2003; Ogura *et al.*, 1988).

The purpose of this study was to assess the susceptibility of MEG-01 cells to *A. phagocytophilum* infection and to evaluate further the pathogen effects on proplatelet formation and platelet production. We hypothesized that MEG-01 cells would be susceptible to infection and that infection might alter platelet production and thus contribute to infection-induced thrombocytopenia. Our findings suggest that MK lineage cells are indeed susceptible to *A. phagocytophilum* infection. However, infection does not significantly alter proplatelet formation or platelet production.

METHODS

Megakaryocytic cell line culture. A human megakaryocytic cell culture line (MEG-01 cells) was obtained from ATCC and propagated in RPMI 1640 (Invitrogen) containing 10% heat-inactivated fetal bovine serum (FBS) and 2 mM L-glutamine. Cells were maintained at 37 °C with 5% CO₂ as described by Ogura *et al.* (1985).

Growth of bacteria and infection of MEG-01 cells with *A. phagocytophilum*. *A. phagocytophilum* (NCH-1 isolate; Goodman *et al.*, 1996; Telford *et al.*, 1996) was maintained in the human promyelocytic cell line (HL60 cells) as described previously (Borjesson *et al.*, 2005b). In brief, HL60 cells were cultured in Iscove's modified Dulbecco's medium (HyClone) containing 20% heat-inactivated FBS at 37 °C with 5% CO₂. *A. phagocytophilum* was harvested from infected HL60 cells as described previously (Borjesson *et al.*, 2005b), resuspended in RPMI 1640 and immediately inoculated into MEG-01 cell cultures (5×10^5 cells ml⁻¹). Cells were evaluated for infection daily by cytofuge preparation and Protocol Hema3 staining (ThermoFisher Scientific). For infection kinetics experiments, DNA encoding the primary outer-membrane protein of *A. phagocytophilum* (p44) was quantified using real-time PCR (ABI Prism 7700 Sequence Detector; Applied Biosystems) as described previously (Borjesson *et al.*, 2002).

Electron microscopy. Infected and uninfected MEG-01 cells (3×10^6) were pelleted in a microfuge at 1200 r.p.m. for 5 min. Cells were fixed in 2 ml 2.5% glutaraldehyde in PBS (pH 7.4) for 2 h. The cells were washed three times in PBS for 5–10 min each, pelleted at 1200 r.p.m. for 5 min and fixed in 1% osmium tetroxide in PBS

for 5 min. The samples were then processed for electron microscopy as described by White (2005).

Flow cytometric detection of MEG-01 cell-surface receptors.

Uninfected MEG-01 cells (1×10^5) were diluted in buffer (1.0% FBS in PBS). Cells were incubated with PL1 (215 antibody, mouse IgG2a; Santa Cruz Biotechnology), CSLEX1 (mouse IgM; BD Biosciences), PL2 (rat IgM; BD Biosciences) or the respective isotype-matched controls for 30 min (all at 10 µg ml⁻¹) at room temperature. The cells were washed once and incubated with FITC- or phycoerythrin-conjugated goat anti-mouse IgG, anti-mouse IgM or anti-rat IgM in the dark for 30 min at room temperature. The cells were washed once and resuspended in buffer containing 1% paraformaldehyde. All samples were analysed on a FACSCanto flow cytometer (BD Biosciences) using CellQuest software.

PL1 blocking. Infected MEG-01 cells (5×10^5) were plated in 12-well tissue culture plates and diluted with uninfected MEG-01 cells until their infection burden was 5%, as assessed by stained cytofuge preparations. MEG-01 cells were incubated for 1 h with either no antibody (positive control), a blocking antibody to PSGL-1 (2 µg PL1 ml⁻¹; Ancell) or the isotype-matched control (anti-mouse IgG1). All conditions were run in triplicate. The cells were washed twice, a time 0 sample was obtained and the remaining wells were incubated at 37 °C with 5% CO₂. The infection burden was monitored by cytofuge preparation and quantitative PCR (to detect *A. phagocytophilum* p44 DNA) on days 0, 3 and 5 post-infection (p.i.).

RT-PCR for gene expression. Total RNA was isolated from cultured MEG-01 cells as described previously (Borjesson *et al.*, 2005b). In brief, 600 µl RLT buffer (Qiagen; containing 10 µl 2-mercaptoethanol ml⁻¹) was added directly to the wells containing MEG-01 cells. The samples were mixed by pipetting and transferred to 1.5 ml RNase-free Eppendorf tubes. Samples were further homogenized by centrifugation in QIAshredder spin columns (Qiagen) at 18 000 g, followed by RNA isolation using an RNeasy mini kit (Qiagen). Samples were treated with DNase (Invitrogen). DNA digestion was confirmed by RT-PCR with primers targeting human β-actin in the presence and absence of reverse transcriptase. Samples were normalized for total RNA concentrations using TaqMan β-actin detection reagents (Applied Biosystems) on a MyiQ Single-Colour Real-Time PCR Detection System (Bio-Rad). cDNA was generated from normalized RNA samples using a SuperScript III First-strand Synthesis System for RT-PCR (Invitrogen). cDNA samples were used as templates in PCRs targeting human sialyl transferases, α1,3-fucosyltransferases, PSGL-1 and β-actin, as described previously (Reneer *et al.*, 2006).

MEG-01 induction and proliferation. MEG-01 cells were induced to differentiate using CH11 (anti-Fas agonistic monoclonal antibody; Upstate Biotechnology) (Chuang & Schleef, 2001; Clarke *et al.*, 2003; Ogura *et al.*, 1988). In brief, MEG-01 cells were plated in 12-well tissue culture plates (Corning Costar) at a final concentration of 3×10^5 cells per well. Cells were incubated with CH11 (50 ng ml⁻¹) for 8–96 h.

The proliferation of MEG-01 cells in response to *A. phagocytophilum* infection was examined. Cell-free *A. phagocytophilum* was inoculated into induced (72 h) and uninduced MEG-01 cells (ratio of 2:1, infected HL60 cells: MEG-01 cells, ~20–50 *A. phagocytophilum* per MEG-01 cell), as described previously (Borjesson *et al.*, 2005b). Plates were centrifuged at 380 g for 8 min to synchronize bacterial uptake (Borjesson *et al.*, 2005b). Culture plates were incubated at 37 °C in a humidified incubator with 5% CO₂ for up to 96 h. Plates were sampled at 24, 48, 72 and 96 h p.i. In order to collect all cells, non-adherent cells and medium supernatant were collected, after which 100 µl trypsin/0.25% EDTA (Invitrogen) was added to the wells and allowed to incubate for 2–4 min. The supernatant was then returned to the well, the well

contents were mixed, and all cells and medium were removed. Wells were washed with PBS and visualized using an inverted microscope to assure complete cell collection. Cells were gently vortexed and aliquots were removed for counting using a haemocytometer. Samples were run in triplicate. Counts were repeated and the mean calculated.

MEG-01 proplatelet formation and platelet production. Platelet production by MEG-01 cells was evaluated in two steps: enumerating the intermediate proplatelet phenotype and enumerating functional platelet yield (Chuang & Schleef, 2001; Clarke *et al.*, 2003; Ogura *et al.*, 1988). In brief, infected and uninfected MEG-01 cells were transferred to tissue culture plates (Corning Costar) at a low density (4×10^4 cells ml^{-1}). Cells were visualized every 24 h until cells in infected wells were at least 40–50 % infected. Half of the infected and half of the uninfected wells were induced to differentiate with CH11. The total number of proplatelet-bearing MEG-01 cells per 500 cells (as indicated by distinctive thin cytoplasmic processes) was enumerated using phase microscopy on an inverted microscope at 24 h post-induction (Clarke *et al.*, 2003). Proplatelet formation was compared between infected and uninfected, and between induced and uninduced MEG-01 cells.

Platelet production from MEG-01 cells was enumerated as described previously by Battinelli *et al.* (2001), Clarke *et al.* (2003) and Takeuchi *et al.* (1998) with minor alterations. Culture supernatants containing platelets from infected and uninfected MEG-01 cells were fixed in the wells with 1 % paraformaldehyde (Sigma-Aldrich) for 30 min. The well contents were collected and separated by centrifugation at 300 *g* for 2 min to pellet the MEG-01 cells, leaving the platelets in the supernatant. The number of MEG-01 cells was determined using a haemocytometer at the time of platelet enumeration. The supernatant was then centrifuged at 300 *g* for 15 min and resuspended in 100 μl PBS. Platelets were stained with 25 μl mouse anti-human mAb to detect α_{IIb} of the $\alpha_{\text{IIb}}\beta_3$ (GPIIb/IIIa) platelet integrin complex (phycoerythrin-conjugated CD41; Ancell). For accurate enumeration, suspended platelets were mixed with TruCount beads (BD Biosciences) according to the manufacturer's instructions. All samples were analysed on a FACSCanto flow cytometer using CellQuest software. Platelets were gated on characteristic forward and side scatter and on positive CD41 expression.

Statistical analysis. Statistical analysis was performed using Student's *t*-test (Microsoft Excel 2002). A *P* value of <0.05 was considered significant.

RESULTS AND DISCUSSION

A. phagocytophilum infects a human megakaryocytic cell line

Host-cell tropism has been a defining biological feature of pathogens in the family *Anaplasmataceae*. Members of this family infect monocytes (e.g. *Ehrlichia chaffeensis*, *Ehrlichia muris* and *Ehrlichia canis*), granulocytes (e.g. *A. phagocytophilum* and *Ehrlichia ewingii*), red blood cells (*Anaplasma marginale*) and platelets (*Anaplasma platys*). Recently, potential host cells have expanded to include endothelial cells (Munderloh *et al.*, 2004), early haematopoietic precursor cells, CD34⁺ cells (Klein *et al.*, 1997) and haematopoietic precursors differentiated along granulocytic and monocytic lineages (Klein *et al.*, 1997).

In our study, *A. phagocytophilum* readily infected megakaryoblastic MEG-01 cells (Fig. 1a, b). Infection kinetics, as assessed by visualization and quantitative PCR, paralleled pathogen growth kinetics in the human myelocytic HL60 cells (Fig. 1c). Our findings expand the host-cell range for this pathogen to include cells of the megakaryocyte lineage and suggest the potential for a broad array of susceptible host cells in the bone marrow. Indeed, in the mouse model of infection, bone marrow is consistently infected as measured by quantitative PCR (Hodzic *et al.*, 2001). Given the cytopenias associated with this disease, even passing infection of cells within the haematopoie-

Fig. 1. *A. phagocytophilum* infects cells of the megakaryocytic lineage (MEG-01). (a) Cytofuge smear of MEG-01 cells with intracellular bacterial colonies (morulae, arrow; stained with Protocol Hema-3). Bar, 0.01 mm. (b) Electron micrograph of an *A. phagocytophilum*-infected MEG-01 cell. The arrows depict typical bacterial colonies with individual bacteria forming a morula structure within a cytoplasmic vacuole. A, cell nucleus. Bar, 5.0 μm . (c) Kinetics of bacterial replication in MEG-01 cells. Quantitative PCR for the bacterial outer-membrane protein *p44* gene showed that bacterial replication in MEG-01 cells parallels that in the prototype human granulocytic leukaemia cell line HL60.

tic microenvironment may alter cell proliferation and differentiation.

***A. phagocytophilum* uses sialylated ligands for optimal infection**

The ability of *A. phagocytophilum* to bind and enter host myeloid cells has been associated with host-cell surface expression of PSGL-1 and sialylated and α 1,3-fucosylated glycans including sLe^x antigen (Goodman *et al.*, 1999; Herron *et al.*, 2000; Yago *et al.*, 2003). Optimal host-cell infection occurs with the cooperative binding of at least two bacterial adhesins to these receptors (Yago *et al.*, 2003). As such, we assessed the surface expression of PSGL-1 on MEG-01 cells. Initial flow cytometric analysis of MEG-01 cell-surface receptors revealed moderate to high surface expression of PSGL-1 using mAb PL1 (Fig. 2a). PL1 blocks *A. phagocytophilum* binding to human PSGL-1 N terminus-modelled glycopeptides and to PSGL-1 on human neutrophil and HL60 cell surfaces (Herron *et al.*, 2000; Li *et al.*, 1996; Yago *et al.*, 2003). Likewise, PL1 successfully blocked *A. phagocytophilum* infection of MEG-01 cells (Fig. 3a, b).

Prolonged culture of leukaemic cells may result in altered surface molecule expression (Wagers *et al.*, 1998). In this study, two subsets of MEG-01 cells emerged that showed relative resistance to *A. phagocytophilum* infection (~20 passages over 6–8 weeks). Infection kinetics differed in lag time from inoculation to visible infection and in the maximal percentage of infected cells as assessed by cytospin preparations (90% infected cells decreased to $\leq$ 25%). These cells

were analysed to assess ligand modifications that best defined the susceptibility of MEG-01 cells to *A. phagocytophilum* infection. Flow cytometric analysis revealed an absence of PL1 binding to the resistant subpopulations (Fig. 2b). Further assessment showed moderate binding of PL2 (Fig. 2c) and CSLEX1 (Fig. 2d). The monoclonal antibody PL2 targets a membrane-proximal region of PSGL-1, whilst CSLEX1 is a sLe^x-specific monoclonal antibody that recognizes sialic acid in an α 2,3-linkage with galactose. RT-PCR was performed to determine whether the absence of PL1 binding was associated with loss of the PSGL-1 transcript. PSGL-1 and appropriate fucosyltransferase (IV and VII) transcripts were present (data not shown). The low to absent PL1 binding to MEG-01 cells suggested an alteration in the PSGL-1 N terminus that normally allows PL1 to recognize the ligand. Of two MEG-01 clones that showed no PL1 binding (Fig. 2b), one achieved infection rates of up to 25%, whilst the other was only 5% infected. Interestingly, the more highly infected of these two clones had a greater level of CSLEX1 binding (Fig. 2d). Together, these findings suggest that the most efficient infection of MEG-01 cells by *A. phagocytophilum* is dependent on surface expression of sLe^x and PSGL-1 by the host cell. These findings parallel our recent report of a laboratory-adapted pathogen strain, NCH-1A (Reneer *et al.*, 2006).

Pathogen isolates vary in their ability to accommodate different host-cell determinants. The NCH-1 strain used in this study has shown plasticity in its ability to infect cells using modified host-cell receptors (Reneer *et al.*, 2006). Upon passage in HL60 cells that are defective for

Fig. 2. *A. phagocytophilum* infection of MEG-01 cells is more efficient in cells that express binding sites for PL1 and CSLEX. (a) The original MEG-01 clone expressed high antibody binding of PL1. (b–d) Two clones of relatively infection-resistant MEG-01 cells were evaluated by flow cytometry to determine surface structure modifications that conferred infection resistance in MEG-01 cells. Isotype control antibodies are shown shaded in grey in each histogram. The solid black lines represent a MEG-01 clone in which ~25% of cells became infected with *A. phagocytophilum*, whilst the dashed line represents a MEG-01 clone that was essentially resistant to infection (infection of ~5% of cells). (b) PL1 did not bind to either cell clone. (c) PL2 bound to both clones; however, these antibodies did not define infection susceptibility as the clone with the highest resistance to infection showed higher mean channel fluorescence compared with the more susceptible clone. (d) Cells with increased infection susceptibility showed increased binding to the anti-sLe^x antibody CSLEX1.

Fig. 3. *A. phagocytophilum* infection of PSGL-1-expressing MEG-01 cells is significantly blocked by anti-PSGL-1 antibody (PL1). (a) *A. phagocytophilum* p44 DNA copy number was significantly lower on days 3 and 5 after pathogen inoculation in MEG-01 cells incubated with the PSGL-1 blocking antibody PL1 compared with cells incubated with no antibody or an isotype control antibody (* $P < 0.05$). (b) Visual inspection of the cells corroborated quantitative PCR findings. By day 5 p.i., >90% of MEG-01 cells incubated with either no antibody or with an isotype control antibody were infected, but <8% of MEG-01 cells incubated with PL1 were infected.

sialyltransferase and/or $\alpha 1,3$ -fucosyltransferase activity and therefore cannot produce sLe^x, subpopulations of NCH-1 emerge that show reduced dependency on sLe^x and, consequently, PSGL-1 (Reneer *et al.*, 2006). We have also observed this phenomenon for the HGE1 and HZ strains (Sarkar *et al.*, 2007). Here, we demonstrated that *A. phagocytophilum* could invade MEG-01 cells in a sLe^x-modified PSGL-1-independent manner similar to that observed for HL60 cells. However, as observed for infection of HL60 cells, optimal infection in the absence of the PL1 determinant apparently still involved sLe^x, perhaps involving sLe^x that decorates other selectin ligands expressed on MEG-01 cell surfaces.

Infected MEG-01 cells show decreased proliferation

Empirically, we noted that cultured, infected MEG-01 cells appeared to decrease in number over time compared with their uninfected counterparts. Thus, proliferation of infected MEG-01 cells was examined. Infected, uninduced MEG-01 cells were reduced in number by approximately 25% compared with uninfected, uninduced cells at 24, 48 and 72 h p.i. (data not shown). As noted by others,

induction of MEG-01 cells reduces cell proliferation by promoting differentiation (Clarke *et al.*, 2003). Compared with uninfected, induced cells, infection further reduced induced cell numbers to approximately 50% of the uninfected control cells. Although the mechanism underlying this pathogen-induced alteration was not elucidated, considerations include the induction of apoptosis or alternative disruption of cell proliferation. *A. phagocytophilum* induces a similar alteration in immortalized HL60 cells attributed to a dysfunctional G1-to-S transition with the failure of multiple cell-cycle and apoptosis regulatory events (Bedner *et al.*, 1998; Hsieh *et al.*, 1997). However, *A. phagocytophilum* delays apoptosis of its host neutrophil (Ge & Rikihisa, 2006; Yoshiie *et al.*, 2000). Therefore, given that infection-induced alterations in cell number are likely to be an *in vitro* phenomenon associated with pathogen growth in leukaemic cell lines, we did not pursue this alteration other than to correct for cell number while assessing proplatelet formation and platelet production.

Infection does not alter proplatelet formation or PLP production

Thrombocytopenia is a consistent characteristic of infection with *A. phagocytophilum* in a variety of susceptible species (Bakken *et al.*, 1996; Borjesson *et al.*, 2001; Lester *et al.*, 2005; Madigan & Gribble, 1987; Pusterla *et al.*, 1999). Most strains of *A. phagocytophilum* do not appear to infect or interact directly with platelets (Borjesson *et al.*, 2005a). Antibody-mediated haemolytic anaemia with concurrent thrombocytopenia has been reported to be associated with *A. phagocytophilum* infection in a dog (Bexfield *et al.*, 2005), and platelet autoantibodies have been described in the serum of some human GA patients (Wong & Thomas, 1998). None the less, in most patients and hosts, there is no evidence of antibody-mediated (adaptive) immune-mediated disease. In a mouse model of infection, neither antibody-mediated destruction nor splenic sequestration of platelets was responsible for the acute thrombocytopenia seen with infection (Borjesson *et al.*, 2001).

MEG-01 cells were utilized as a model to assess whether direct infection of MKs might lead to decreased platelet production and thereby be one mechanism of infection-induced thrombocytopenia. Induction of MEG-01 cells with CH11 led to a significant increase in proplatelet formation compared with uninduced cells ($P < 0.001$; Fig. 4a–e and reference Clarke *et al.*, 2003). There was no significant difference ($P = 0.57$) between proplatelet formation in infected and uninfected MEG-01 cells 24 h after induction (Fig. 4e). Similarly, infection did not alter spontaneous proplatelet formation in the uninduced MEG-01 cells ($P = 0.29$, Fig. 4e).

Platelets or PLPs are produced spontaneously by MEG-01 cells (Battinelli *et al.*, 2001; Chuang & Schleef, 2001; Clarke *et al.*, 2003; Takeuchi *et al.*, 1998). Cultured PLPs have characteristic platelet forward and side scatter as assessed by flow cytometry, and display platelet-like shape changes

Fig. 4. Induction of differentiation and apoptosis in MEG-01 cells (using CH11) results in increased proplatelet formation in uninfected and *A. phagocytophilum*-infected MEG-01 cells. Phase-contrast micrographs (magnification $\times 400$) showing uninduced, uninfected MEG-01 cells (a), induced, uninfected MEG-01 cells [note the long, cellular, cytoplasmic extensions (proplatelets)] (b), uninduced, infected MEG-01 cells (c), and induced, infected MEG-01 cells (d). Induction resulted in proplatelet formation. (e) Minimum, maximum, median, and 25 and 75% quartiles of MEG-01 cells expressed as number of cells with proplatelet extensions per 500 cells counted per culture well under all four conditions. The data represent three replicates. Induction led to significantly higher numbers of proplatelet extensions (asterisks indicate significant difference compared with uninduced controls); however, MEG-01 cell infection with *A. phagocytophilum* did not affect proplatelet formation.

and aggregation in response to platelet agonists (Battinelli *et al.*, 2001; Clarke *et al.*, 2003; Takeuchi *et al.*, 1998). They also express surface platelet receptors including GPIIb/III_a (Takeuchi *et al.*, 1998). Initial evaluation in our laboratory confirmed that PLPs display characteristic forward and side scatter by flow cytometry, undergo a characteristic shift in side scatter in response to ADP and express GPIIb/III_a on their surface (data not shown).

As with proplatelet formation, infection of MEG-01 cells did not significantly alter PLP formation ($P=0.53$; Fig. 5). Early replicates of this experiment were performed with MEG-01 cells that became highly infected (at least 60% infected as assessed by cytospin preparations), whilst later replicates were performed with MEG-01 cells that were relatively resistant to *A. phagocytophilum* infection. The lack of effect of *A. phagocytophilum* infection on PLP production was consistent across these varying degrees of infection.

Conclusion

In this study, we have shown that platelet progenitors do become infected with *A. phagocytophilum*, and that infection susceptibility is determined by sialylated ligands similar to those that permit granulocyte infection. However, infection of MEG-01 cells, in and of itself, does not directly alter proplatelet formation and platelet production. Similarly, proplatelet formation and platelet production do not change, regardless of pathogen burden. This suggests that, although haematopoietic cells including MKs are susceptible to infection, infection-induced thrombocytopenia may not be a direct effect of intracellular pathogen. These findings are

Fig. 5. Infection does not alter MEG-01 production of platelets. Depicted are the minimum, maximum, median, and 25 and 75% quartiles of the absolute numbers of platelets produced per 2×10^5 MEG-01 cells, as detected by flow cytometric analysis using TruCount beads, of infected and uninfected MEG-01 cells. The data represent four replicates. MEG-01 cells infected with *A. phagocytophilum* showed no significant difference in platelet production compared with uninfected cells.

compatible with research that suggests that disease severity in the mouse is independent of pathogen burden and is related instead to activation of the innate immune system (Scorpio *et al.*, 2004, 2005). If disrupted haematopoiesis contributes to infection-induced cytopenias, alterations in cytokine/chemokine regulation or stromal cell infection/disruption may supersede direct pathogen effects on megakaryopoiesis, haematopoiesis and differentiation.

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