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RESEARCH ARTICLE

Cellular metabolism and IL-6 concentrations during stimulated inflammation in primary fibroblasts from small and large dog breeds as they age

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ABSTRACT

The immune system undergoes marked changes during aging characterized by a state of chronic, low-grade inflammation termed 'inflammaging'. We explore this phenomenon in domestic dogs, which are the most morphologically and physiologically diverse group of mammals, with the widest range in body sizes for a single species. Additionally, smaller dogs tend to live significantly longer than larger dogs across all breeds. Body size is intricately linked to mass-specific metabolism and aging rates, which suggests that dogs are exemplary for studies in inflammaging. Dermal fibroblast cells play an important role in skin inflammation, making them a good model for inflammatory patterns across dog breed, body sizes and ages. Here, we examined aerobic and glycolytic cellular metabolism, and IL-6 concentrations in primary fibroblast cells isolated from small and large dog breeds, that were either recently born puppies or old dogs after death. We found no differences in cellular metabolism when isolated fibroblasts were treated with lipopolysaccharide (LPS) from *Escherichia coli* to stimulate an inflammatory phenotype. Unlike responses observed in mice and humans, there was a less drastic amplification of IL-6 concentration after LPS treatment in the geriatric population of dogs compared with recently born dogs. In young dogs, we also found evidence that untreated fibroblasts from large breeds had significantly lower IL-6 concentrations than observed for smaller breeds. This implies that the patterns of inflammaging in dogs may be distinct and different from other mammals commonly studied.

KEY WORDS: Body mass, Inflammaging, Lifespan

INTRODUCTION

Physiological aging, which is difficult to define biologically, describes the decrease in physiological function and fitness with age (Ricklefs, 2010). It is progressive, endogenously derived, irreversible and expands across multiple processes and systems within an organism (Cohen, 2018). Owing to the accumulation of deleterious traits in its forward progress (Sanz et al., 2006), every aspect of an organism's phenotype is modified during aging (Kirkwood 2005); thus, lifespan is limited by the rate of an organism's aging. Various processes, including accumulation of oxidative damage, increases in inflammation, telomere shortening, mitochondrial dysfunction and misfolded proteins may affect the rate of aging (Cohen, 2018). Age-related physiological changes to the immune system are considered a main culprit of co-morbidities in old age and are often associated with life expectancy and survival across species (Cossarizza et al., 1997; Strasser et al., 2000). A decline in mitochondrial quality has been linked to specific mechanisms of aging including cellular dysfunction and inflammation (Sun et al., 2016). Mitochondria dysfunction with age can lead to activation of NIrp3 inflammasome, a multiprotein complex that leads to the activation of proinflammatory cytokines IL-1β and IL-18, and the induction of chronic reactive oxygen species (ROS) production (Franceschi and Campisi, 2014). Similarly, the immune system undergoes marked changes during aging characterized by a state of chronic, low-grade inflammation: a process called 'inflammaging' (Franceschi and Campisi, 2014). Specifically, the aging process activates inflammatory molecules, including cytokines, inevitably resulting in systemic inflammation (Baylis et al., 2013). Pro-inflammatory cytokines such as interleukin (IL)-1β and IL-6 in particular are termed the 'cytokines of gerontology' (Franceschi et al., 2000). These inflammatory molecules contribute to inducible, innate immune responses and their release is triggered during immune challenges (Lee, 2006; Zimmerman et al., 2014). For example, IL-6 induces B cell differentiation, activates T cells and thymocytes, and activates acute phase proteins, among other immunological roles (Yamashita et al., 1994). Mechanistically, inflammaging seems to be the consequence of cumulative lifetime exposure to antigenic load (Baylis et al., 2013). It can be caused by accumulation of pro-inflammatory tissue damage, accumulation of pathogens due to an ill-functioning immune system, dysfunctional host cells, to name a few (López-Otín et al., 2013).

Body size is closely related to lifespan and aging rates so that, typically, larger mammals are longer lived than smaller mammals (Promislow, 1993). Because the maintenance of the immune system is a metabolic process, we would expect body mass to have an effect on immunity, particularly in innate responses (Downs et al., 2020; Dingli and Pacheco, 2006). For example, constitutive concentrations of neutrophils, a white blood cell recruited to sites of infection early during an immune response that help eliminate bacterial challenges and the propagation of subsequent immune defense (Kolaczkowska and Kubes, 2013), increases disproportionately with body mass in mammals (Downs et al., 2020). The relationship between a trait and body size is also connected with life history evolution of that species because an individual can only grow large by having an extended maturation period to grow for longer, which is often associated with increased longevity (Harrison, 2017). Much research in ecoimmunology has focused on exploring the prediction that larger animals that tend to be longer-lived would have a higher investment in safety because they live longer (Lee, 2006).

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Domestic dogs are a morphologically and physiologically diverse group of mammals. Underlying observed morphological differences there must be cellular-level disparities that could lead to elucidating aging rates and lifespan discrepancies across dog breeds. Smaller dogs tend to live significantly longer than larger dogs across all breeds (Jimenez, 2016). Smaller dogs also have lower cancer risks (Michell, 1999), and demonstrate lower prevalence of age-related diseases such as cataracts (Urfer et al., 2011), implying slower rates of aging. Often, the aging literature refers to the GH–IGF-1 (growth hormone–insulin-like growth factor 1) pathway as the most conserved aging pathway across species (López-Otín et al., 2013; Cohen, 2018). The somatotrophic axis in mammals is composed of growth hormone, produced by the anterior pituitary and IGF-1, which is produced in response to GH in many cell types. This pathway informs cells on the presence of glucose. Insulin is involved in cell growth, and glucose homeostasis (Partridge and Gems, 2002) whereas IGF-1 is thought to influence cell growth, differentiation and survival (Dantzer and Swanson, 2012). Insulin and IGF-1 concentration seem to be negatively correlated with lifespan in several mouse strains selected for disparate lifespans (Fontana et al., 2010; Tian et al., 2017), where mice selected for dwarfism due to lack of GH production and a reduction of IGF-1 in plasma had longer lives than their control counterparts (Dantzer and Swanson, 2012). A single *IGF1* haplotype seems to substantially contribute to size variation in dogs (Sutter et al., 2007), and serum IGF-1 concentration in small dogs is reduced relative to that found in large breeds, providing a potential link to their longer lives (Eigenmann et al., 1988; Greer et al., 2007).

Particularly in human studies, aging has been demonstrated to increase levels of IL-6 and tumor necrosis factor alpha (TNF-α) and decrease IGF-1 levels (Xia et al., 2016), a pattern unexplored in dogs. IL-6 concentrations significantly predict mortality and predicted 2-year survival in very old humans, and seem to be positively associated with other age-related diseases such as type 2 diabetes, cardiovascular disease, Parkinson's disease, cancer and arthritis (Wikby et al., 2006; Baylis et al., 2013; Xia et al., 2016). Thus, the increases of IL-6 associated with an aging immune system may contribute to increases in incidences of disease and cancer (Kearns et al., 1999; Wikby et al., 2006).

To what degree donor body mass and/or age influence the inflammatory response *in vitro* is unknown for any mammal, much less for dogs, but there is no question that inflammatory dysfunction is a hallmark of aging (Franceschi and Campisi, 2014). In aging dogs, there seems to be marked changes in T-cell immunity, including changes in the number, and subsets of T-cells, which may lead to a reduced ability to respond to non-specific mitogens (Day, 2010). Older dogs also seem to have more immunoglobins than puppies (Day, 2010). Older female dogs seem to demonstrate increases in IL-1 activity compared with younger dogs (Day, 2010). There have been studies in dogs demonstrating decreases in mitogen stimulation (Greeley et al., 1996), number of white blood cells, and immature neutrophils (Strasser et al., 1993) with increasing age. However, none of these studies have addressed whether these changes differ between the different breed sizes as they age.

Here, we examine aerobic and glycolytic cellular metabolism, and IL-6 concentration in primary fibroblast cells isolated from small and large breed, recently born and old deceased dogs. Isolated fibroblasts were then treated with lipopolysaccharide (LPS) from *Escherichia coli*. LPS molecules from the cell wall of Gram-negative bacteria are an immunostimulatory agent that initiates synthesis and release of proinflammatory cytokines, including IL-6 (Agarwal et al., 1995). We predicted that (1) aerobic and anaerobic metabolism would increase with LPS treatment; (2) that small breeds (longer lived) would have a better capacity to deal with LPS treatment, either through smaller increases in aerobic and anaerobic metabolism or no change to spare respiratory capacity, compared with large breed dogs; (3) that recently born dogs would be better suited to deal with an LPS treatment; and (4) IL-6 concentrations would be greater in deceased dogs compared with recently born dogs. Expression of IL-6 can be induced in different cell types, including fibroblasts, monocytes, T cells, B cells, epidermal cells, and even in tumor cells (Yamashita et al., 1994). Because fibroblasts are the main cell type responsible for wound healing (including inflammation, re-forming the epithelial matrix and remodeling), these cells play an important role in skin inflammation, and as such, are a good study model to determine inflammatory patterns in dogs (Tsuchiya et al., 2015).

MATERIALS AND METHODS

Isolation of dog primary fibroblasts

We isolated primary fibroblast cells from recently born and deceased dogs of two size classes. The small breed size class was composed of breeds with an adult body mass of 15 kg or less, and the large breed size class included breeds or mixes with an adult body mass of 20 kg or more These size classes are based on American Kennel club (AKC) standards of each breed, and described in Jimenez (2016). Small breed recently born puppies (N=45) were on average 3.4 days old at the time of sample collection. Small breed deceased dogs (N=12) were on average 13.7 years old at the time of sample collection. Large breed puppies (N=74) were on average 9.6 days old at the time of sample collection. Large breed deceased dogs (N=15) were on average 11.9 years old at the time of sample collection. Samples from recently born puppies were obtained from routine tail docks, ear clips and dew claw removals performed at veterinarian offices in Central New York and Michigan. Samples from deceased dogs were collected from ear clips immediately after euthanasia in Central New York. The samples were placed in cold transfer medium [Dulbecco's modified Eagle medium (DMEM), with 4.5 g l^{-1} glucose, sodium pyruvate, and 4 mmol l^{-1} L-glutamine supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (100 U ml^{-1} penicillin/streptomycin], containing 10 mmol l^{-1} HEPES) and transferred to Colgate University. To isolate primary fibroblast cells, skin samples were sterilized in 20% bleach and 70% ethanol. Once any fat and bone were removed, skin was minced and incubated in sterile 0.5% collagenase type 2 (Worthington Chemicals, cat. no. LS004176) overnight in an atmosphere of 37°C, 5% CO_2 and 5% O_2. After incubation, the collagenase mixture was filtered through a 20 µm sterile mesh, and centrifuged at 1000 rpm for 5 min. The resulting supernatant was removed, and the pellet was resuspended with 7 ml mammal medium [DMEM with 4.5 g l^{-1} glucose, sodium pyruvate, and 4 mmol l^{-1} L-glutamine supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (100 U ml^{-1} penicillin/streptomycin)]. Cells were grown in Corning T-25 culture flasks at 37°C in an atmosphere of 5% O_2 and 5% CO_2. When cells reached 90% confluence, they were trypsinized (0.25%) and cryopreserved at 10^6 cells ml^{-1} in DMEM supplemented with 40% fetal bovine serum and dimethylsulfoxide (DMSO) at a final concentration of 10%. We stored cells in liquid N_2 prior to any experiments. All the procedures within this study were approved by the Colgate University Institutional Care and Use Committee under protocol number 1819-13.

Treatments

Each cell line was resuspended in 7 ml of complete medium (as above) and given 5 days to recover from freezing in an atmosphere of 37°C, 5% O_2 and 5% CO_2. Cells were plated at passage 2 (P2) in a concentration of 10,000 cells per well on a seahorse microculture plate. Cells from each dog were plated in duplicate per treatment (control or LPS). Treated cells were incubated with an LPS from *E. coli* (O111:B4, Sigma Aldrich, cat. no. L2630) at a concentration of 30 µg ml^{-1} for 24 h (Wendell and Stein, 2001; Tabeta et al., 2000), whereas control wells were not exposed to LPS. Immediately following the LPS incubation, oxygen consumption rate (OCR; aerobic metabolic rate) and extracellular acidification rates (ECAR; anaerobic/glycolytic metabolic rate) were performed as described in Patton et al. (2018), and below.

Oxygen consumption rate (OCR) in primary fibroblast cells

OCR was determined using XF-96 FluxPaks from Agilent Technologies. We measured OCRs after cells were equilibrated for 1 h to running medium, which contained 10 mmol l^{-1} glucose, 1 mmol l^{-1} sodium pyruvate and 2 mmol l^{-1} glutamine, pH=7.4. Baseline measurements of OCRs were made three times to establish basal OCR prior to injecting a final well concentration of 2 µmol l^{-1} oligomycin, which inhibits ATP synthesis by blocking the proton channel of the F0 portion of the ATP synthase, causing OCR to fall. Thus, the decrease in OCR from basal levels represents ATP coupled respiration, whereas the remaining OCR is attributed to O_2 consumption required to overcome the natural proton leak across the inner mitochondrial membrane plus any non-mitochondrial O_2 consumption. We then injected a well concentration of 0.125 µmol l^{-1} carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), an uncoupling agent that disrupts ATP synthesis by collapsing the proton gradient across the mitochondrial membrane leading to uncoupled consumption of energy and O_2 without generating ATP, providing a theoretical maximal OCR. Finally, we injected a final well concentration of 0.5 µmol l^{-1} antimycin A, a complex III inhibitor and rotenone, a complex I inhibitor. This combination stops mitochondrial respiration and enables non-mitochondrial respiration to be evaluated (Gerencser et al., 2009; Brand and Nicholls, 2011; Hill et al., 2012). In addition to the OCR parameters we measured directly (as listed above), we also calculated spare respiratory capacity as the difference between maximal OCR and basal OCR, and ATP coupling efficiency as the percentage of basal OCR accounted for by ATP-coupled respiration (Divakaruni et al., 2014). After measurements were completed, we used a 1:200 concentration of CyQUANT dye to quantify final counts of cells in each well (Jones et al., 2001) and normalized all rates to 20,000 cells.

Extracellular acidification rate (ECAR) in primary fibroblast cells

ECAR values were measured in units of mpH, which is the change in pH in the medium surrounding the cells due to proton flux in glycolysis. Measurements of ECAR were performed after the cells were equilibrated to running medium for 1 h. Running medium contained no glucose and 2 mmol l^{-1} L-glutamine in all experiments, pH=7.4. Baseline rates were measured three times prior to any injections. We injected a final well concentration of 10 mmol l^{-1} glucose into medium surrounding cells, which provides a measure of glycolytic rate, and then we injected a final well concentration of 2 µmol l^{-1} oligomycin, giving us an estimate of glycolytic capacity in cells. Finally, we injected a final well concentration of 50 mmol l^{-1} 2-DG, a glucose analog that inhibits glycolysis, which provided an estimate of non-glycolytic acidification (Hill et al., 2012). After measurements were completed, we used a 1:200 concentration of CyQUANT dye to quantify final counts of cells in each well (Jones et al., 2001) and normalized all rates to 20,000 cells.

IL-6 assay

Immediately after 24 h of treatment, supernatants from control and LPS-conditioned cells were collected from each individual well (duplicated per dog as stated above) to assess the secretory profile of the pro-inflammatory cytokine IL-6 and were frozen at −20°C until further use. We used a commercially available kit (Canine IL-6 ELISA kit RayBiotech) to quantify IL-6 in 100 µl of supernatants following the manufacturer's protocol. We used a 1:200 concentration of CyQUANT dye to quantify final counts of cells in each well (Jones et al., 2001) and normalized all IL-6 concentrations to 20,000 cells. Intra-assay variability was 11.2–12.5%, whereas inter-assay variability was 13.1%

Statistics

Data from every assay were first tested for normality using a Shapiro–Wilks test. The data were transformed in a manner informed by a Box–Cox power transformation (Box and Cox, 1964). This meant a log transform for all variables except IL-6, which got an inverse-square transformation, i.e. ($1/Y^2$). First, we analyzed all data in a single repeated measures MANOVA to test for differences in the collection of dependent variables across body size (small or large, as stated above), sample type (recently born or deceased) and treatment (control or LPS), their two-way interactions and a three-way interaction. Follow-up analyses were conducted using three-way repeated measures ANOVAs on each dependent variable. For dependent variables that suggested a treatment effect, we fitted mixed effects models using the lmerTest package (Kuznetsova et al., 2017) for R (https://www.r-project.org/) to quantify the effect. When conducting the analyses, we accounted for the repeated measures design by including a random intercept for subjects. Best-subset model selection according to the Bayesian information criterion (BIC) (Schwarz, 1978) was completed via complete enumeration (Morgan and Tatar, 1972) for each model. BIC penalizes false positives more than false negatives when there are eight or more observations, and is asymptotically consistent. We adjusted the *P*-values in the MANOVA analyses and within each model reported using the approach in Benjamini and Hochberg (1995) rather than using the overly conservative Bonferroni adjustment (Dunn, 1961). This approach allows for the control of the expected proportion of false discoveries instead of the probability of making at least one false discovery, thus preserving power. In the statistical models (see full details at https://figshare.com/s/ee6a5f273a542b703857), we included categorical size, which allowed us to consider the fact that recently born dogs would have a disproportionally small body mass, but could be part of the large size class, noting that this also better matches the goal of the experiment. *Post hoc* testing involved estimating marginal means for specified values of independent variables and comparing these means via statistical contrasts. We performed these *post hoc* tests via the emmeans package for R (https://cran.R-project.org/package=emmeans). Results were considered significant if the adjusted *P*-value was less than 0.05.

Limitations

Owing to the nature of working with pet dogs, our sample collection was limited in several ways. First, the number of recently born dogs

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per breed or group was limited to those breeds that are altered as a breed requirement (tail docks and dew claw removal). The number of euthanized dogs was limited owing to the owners' willingness to provide us ear clips at the time of euthanasia. This collection design also yielded a gap in observations between 0.20 and 6 years of age, hence using sample type as a surrogate for age; we note that this diminishes statistical power to detect the effect of age but we feel it better represents the data available. Secondly, we were not given complete medical charts for any dogs included in this study, thus, we have no records of diet or exercise, which may be variants in OCR, ECAR and background inflammation parameters. At times, veterinarians did not provide sex or weight for recently born dogs either. We do know that none of the dogs included in this study were taking any metabolic, endocrine or nervous system medications, and none were obese. Furthermore, young puppies may not have a fully developed immune system, and older dogs may have a declining immune system and underlying systemic diseases. However, we used primary fibroblast cells to elicit and measure the capabilities of mounting a response at the cellular level.

RESULTS

Previously published work (Jimenez et al., 2018) describes differences associated with size class and age class (sample type). Here, we focused on the differences between control and LPS-treated fibroblasts, as well as the interactive effects of size and sample type with treatment. Full details of statistical models and all MANOVA/ANOVA results can be found on Figshare at: https://figshare.com/s/ee6a5f273a542b703857.

The repeated measures MANOVA showed significant differences for the multivariate analysis in sample type (F=3.4030, P=0.0019), size class (F=4.7953, P=0.0001) and treatment (F=3.4842, P=0.0019) (see table 1 in https://figshare.com/s/ee6a5f273a542b703857). Additionally, there was a statistically significant Sample type×Treatment interaction (F=2.6885, P=0.0109). Thus, we probed each dependent variable separately using repeated measures ANOVA.

Results showed significant unique effects of treatment on non-mitochondrial respiration (P=0.0371; Table 1) and IL-6 concentrations (P<0.0001; Fig. 1 and Table 1). Additionally, the treatment effect did not reach traditional levels of statistical significance for non-glycolytic acidification, total glycolysis, total glycolytic capacity, basal OCR, proton leak, maximal respiration, or spare respiratory capacity through the unique or interaction effects (Table 1).

Full transformed model

Full models were fitted for non-mitochondrial respiration and IL-6 to follow up the statistically significant effect of treatment in the ANOVA analyses for these dependent variables. The non-mitochondrial respiration model required a log transformation and the IL-6 model required an inverse square transformation to ameliorate concerns with assumptions of the linear mixed effects model. Despite significant results in the three-way ANOVA, the treatment effect did not reach traditional levels of statistical significance for non-mitochondrial respiration through the unique effect of treatment (P=0.1298), the Treatment×Sample type interaction (P=0.9657), the Treatment×Size interaction (P=0.8892) or through the Sample type×Size×Treatment three-way interaction (P=0.6822) (Table 2). There was, however, a significant unique treatment effect on IL-6 (P<0.0001), and a significant interaction is the Sample type×Size (P=0.0271), whereas Treatment×Sample type (P=0.0880), Treatment×Size (P=0.7374) and Sample type×Size×Treatment (P=0.1090) interactions did not reach traditional levels of statistical significance (α=0.05). See 'Post hoc Analysis of the Transformed Model' in https://figshare.com/s/ee6a5f273a542b703857 for full details.

Post hoc analysis of the full transformed model for IL-6

Control (background) values of IL-6 showed that fibroblasts from recently born small breed dogs had a significantly higher concentration of IL-6 compared with those from young large breed dogs (P=0.0065). After LPS treatment, we found differences between cells from young and old large breed dogs (P=0.0141), young and old small breed dogs (P=0.0112) and large and small breed recently born dogs (P=0.0021). When treated with LPS, fibroblasts from young small breed dogs (P<0.0001), young large breed dogs (P<0.0001) and old small breed dogs (P=0.0002), but not old large breed dogs (P=0.6028) had higher IL-6 concentrations compared with untreated cells.

Furthermore, cells from old large breed dogs showed a less pronounced treatment effect compared with those from young large breed (P<0.0001), old small breed (P=0.0288) and young small breed (P<0.0001) dogs. There was also a larger treatment effect for cells from old small breed versus young large breed (P<0.0001) dogs. For full details of all these comparisons, see 'Post-hoc Analysis of the Transformed Model' section in: https://figshare.com/s/ee6a5f273a542b703857.

Best subsets model

When selecting independent variables using best subsets regression according to BIC, we identified the best model following hierarchy without including redundant or unnecessary terms. These models supported a 27.83% increase in non-mitochondrial respiration (P=0.0002) and increased IL-6 (P<0.0001) for LPS-treated compared with untreated fibroblasts.

Table 1. ANOVA results after *P*-value adjustment

	Non-glycolytic	Glycolysis	Glycolytic capacity	Basal OCR	Proton leak	Max. respiration	Spare respiratory capacity	Non-mito. respiration	IL-6
Sample type	0.0002*	0.3219	0.0162*	0.0122*	0.0249*	0.0009*	0.0012*	0.0003*	0.6809
Treatment	0.0598	0.5011	0.5640	0.9263	0.7442	0.9626	0.4587	0.0371*	<0.0001*
Size class	0.4493	0.7142	0.5640	0.8399	0.2493	0.9626	0.5118	0.1248	0.1365
Sample type×Treatment	0.6371	0.4552	0.4265	0.8399	0.2493	0.9626	0.6919	0.9626	0.4232
Sample type×Size class	0.5057	0.8629	0.4874	0.5090	0.2493	0.1584	0.0612	0.3423	0.1591
Treatment×Size class	0.2543	0.4552	0.2564	0.8399	0.7442	0.9626	0.4587	0.9626	0.4232
Sample type×Treatment×Size class	0.6371	0.4552	0.4265	0.9263	0.7442	0.9626	0.7628	0.7077	0.0061*

Results that reach traditional levels of statistical significance are marked with an asterisk. Statistics, degrees of freedom and raw *P*-values are reported in https://figshare.com/s/ee6a5f273a542b703857.

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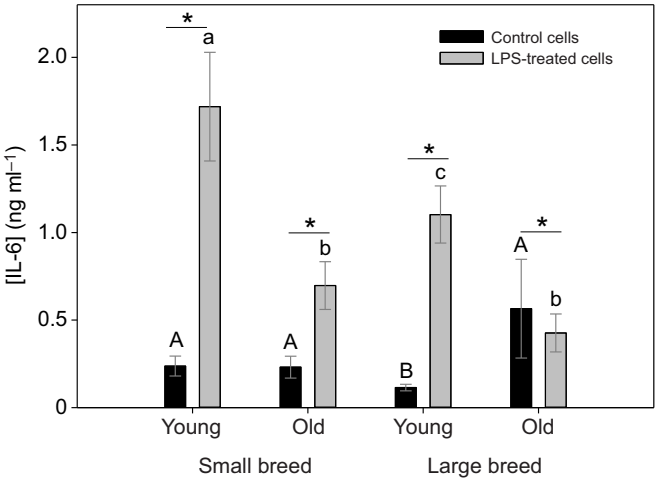

Fig. 1. IL-6 levels in primary fibroblast cells from small and large breed, young and deceased (old) dogs treated with lipopolysaccharide (LPS) from *E. coli*. Isolated fibroblasts were treated with 30 µg/ml LPS for 24 h or not (background/control). After 24 h, IL-6 concentrations (normalized to 20,000 cells for each condition) were significantly different between control and LPS-treated cells (P<0.0001; significant differences indicated by asterisks), in all except the large deceased dogs. Capital letters demonstrate when IL-6 is significantly different at background levels. Lower case letters demonstrate when IL-6 is significantly different after LPS treatment. Bars represent mean± s.e.m. Small breed: young, N=36; old, N=12; large breed: young, N=65; old, N=12.

DISCUSSION

Immunosenescence is well documented in dogs (Day, 2010; Alexander et al., 2018); however, only a handful of studies have investigated pro-inflammatory pathways in older dogs (Day, 2010). To our knowledge, this is the first study that examines the effects of body size, as well as age class in terms of cellular metabolism and response to a pro-inflammatory challenge in the domestic dog system. Constitutive components of the innate immune system are responsible for producing inflammatory cytokines resulting from local inflammation. At the organismal level, this upregulation of the inflammatory response is marked by increases in acute phase proteins in the liver, and costly changes in energy metabolism (Lee, 2006). LPS also stimulates an increase in synthesis of growth factors and cytokines at the cellular level (Pang et al., 1994). Our results do not support our predictions listed in the Introduction, as we found no differences in cellular metabolism with LPS treatment, across body size and age classes (sample type), with the exception of non-mitochondrial respiration. We also found the opposite pattern to that we predicted for IL-6 concentration. Others have found that decreased mitochondrial membrane uncoupling found in large breed dog primary fibroblast cells, coupled with lower potential of β-oxidation and accumulation of acylcarnitines, promotes inflammation (Nicholatos et al., 2019), which follows the pattern in our current study that young large breed dogs seem to have a higher IL-6 concentration.

Life history theory predicts that fast- and slow-paced species should invest differently in inflammatory responses because their cost–benefit structure differs (Lee, 2006). Slow-paced species live longer than fast-paced species and thus benefit more from responding to infections in a manner that limits immunopathology, whereas fast-paced species benefit from clearing infections and maximizing current reproductive output regardless of the long-term costs of the immune response. It follows that species on the ‘fast’ end of the life-history spectrum should have proinflammatory responses that cause more immunopathology, whereas species on the ‘slow’ end should invest in anti-inflammatory responses that reduce immunopathology (Sears et al., 2011). Determining life-history differences in domestic dogs is not clear-cut, as small dog breeds live longer, and have fewer pups per litter – both determinants of a slower pace of life – but also seem to have faster growth rates compared with large breed dogs (Jimenez, 2016) like many faster pace of life animals. IL-6 background concentration showed that recently born small breed dogs had significantly higher IL-6 background concentration compared with recently born large breed dogs, and IL-6 background (control) concentration is similar in deceased small breed dogs, and deceased large breed dogs. However, our sample size for large breed old dogs may have limited these interpretations owing to a lack of statistical power. This pattern of higher IL-6 background concentrations may point to an anti-inflammatory investment strategy in small dogs at the ‘slow’ end of the life history spectrum.

Similar to findings included in this study, other studies have found differences between small and large size classes with respect to glycolytic parameters in dogs, where larger breed puppies have significantly higher glycolytic capacity compared with smaller breeds (Jimenez et al., 2018), although it seems this pattern is related to increased glucose oxidation (Brookes and Jimenez, 2021). LPS treatment has been shown to increase lactate concentrations in the acute monocytic leukaemia cell line compared with controls (Ubanako et al., 2019). Intracellular inflammatory mediators, such as TNF-α directly inhibit insulin signaling (Odegaard and Chawla, 2013), which would alter aerobic metabolism. Neither of these were applicable in our study, as we did not see any changes in cellular

Table 2. Regression results after *P*-value adjustment

	Non-glycolytic	Glycolysis	Glycolytic capacity	Basal OCR	Proton leak	Max. respiration	Spare respiratory capacity	Non-mito. respiration	IL-6
Overall	–	–	–	–	–	–	–	<0.0001*	<0.0001*
Small breed, young	–	–	–	–	–	–	–	–	<0.0001*
Large breed, young	–	–	–	–	–	–	–	–	<0.0001*
Small breed, old	–	–	–	–	–	–	–	–	0.0002*
Large breed, old	–	–	–	–	–	–	–	–	0.6028

The ‘Overall’ row indicates whether the treatment effect was significant in the best-subsets model. Subsequent rows break down results from *post hoc* testing the full models to show significant treatment differences for specific sample type and size classes, averaged across control variables. Note that modelling was only conducted for non-mitochondrial respiration and IL-6, which showed a significant treatment effect in the ANOVA, and we only probed the full transformed model for IL-6 because the treatment effects were not significant in the full transformed model for non-mitochondrial respiration. Results that reach traditional levels of statistical significance are marked with an asterisk. Statistics, degrees of freedom and raw *P*-values are reported in https://figshare.com/s/ee6a5f273a542b703857.

metabolic rates with LPS. However, non-mitochondrial oxygen consumption includes oxygen-consuming oxidases functioning throughout metabolism, such as peroxisomal fatty acid oxidation (Rolfe and Brown, 1997), and we saw an increase in non-mitochondrial respiration in LPS treated cells compared with controls. This could be due to increases in NADH oxidases that seem to be linked to increases in IL-6 concentration (Behrens et al., 2008; Yu et al., 2005).

In dogs, there is little information about the role of IL-6 during the acute phase response due to inflammatory stimuli (Yamashita et al., 1994). In beagle dogs injected with turpentine oil to induce an inflammatory response, IL-6 concentration in blood increased by 4 h after the injection until reaching a maximum at 12 h post injection, and remained higher than levels in the controls up to 6 days after the injection (Yamashita et al., 1994). Stimulated LPS responses in whole blood of aging dogs demonstrated a significant increase in IL-6 concentrations compared with control values, regardless of age, and although their study included dogs of different breeds, there seemed to be no correction of the data for body size (Deitschel et al., 2010). Different dog tissues have demonstrated different abilities to upregulate IL-6 concentrations with peritoneal cells having a more exaggerated response compared with peripheral blood mononuclear cells (PBMCs) isolated from fox terriers and Labrador retrievers, although age did not seem to affect IL-6 concentrations in either tissue or breed (Kearns et al., 1999). Our results are similar to those found in other studies of dogs (Deitschel et al., 2010). Unlike geriatric mice and humans (Tateda et al., 1996; Gabriel et al., 2002), the adult population of dogs exhibited a less drastic amplification of IL-6 concentration after LPS treatment. Rather, cells from younger dogs of both small and large size classes demonstrated a drastic upregulation of IL-6 when treated with LPS. In contrast, background IL-6 data from our study does follow the patterns of geriatric humans and mice when considering just the large dog size class, such that fibroblasts from geriatric large dogs have an increase in background IL-6 compared with cells from their younger counterparts. In humans, IL-6 concentrations are undetectable in young people, but start to increase at age 50–60 and continue to increase so that even healthy centenarians have high levels of IL-6; however, increases in IL-6 in humans have also been demonstrated as predictors of morbidities and mortality in elderly people and are linked to chronic inflammatory diseases (Franceschi et al., 2000; Maggio et al., 2006). Although the main source of inflammation associated with chronic disease and aging is leukocytes, particularly macrophages and monocytes (Sarkar and Fisher, 2006). In Labrador retrievers, C-reactive protein (CRP), which is produced by the liver in response to IL-6 and TNF-α, increased with age (Alexander et al., 2018), similar to our results in old large dogs.

Previous work suggests that DNA oxidative damage (8-OHdg) negatively correlates with mean breed lifespan, so that shorter lived breeds may accumulate more oxidative DNA damage compared with longer lived breeds (Jimenez et al., 2018). Others have also found that plasma 8-OHdg levels increase with age in Labrador retrievers (Alexander et al., 2018). Damaged DNA in the nucleus including DNA double strand breaks (DSBs) commonly accumulated as oxidative damage (Hasty et al., 2003), can stimulate proinflammatory responses (Brzostek-Racine et al., 2011). Acutely, these responses are initiated through communication between the nucleus and the cytoplasm to include activation of nuclear factors, cell surface ligands or intercellular adhesion molecules, leading to an upregulation of pro-inflammatory cytokines (Chatzinikolaou et al., 2014). Chronically, DNA damage due to age-accumulated cellular senescence leads to a phenotype that entails the secretion of cytokines, such as IL-6 and IL-8, promoting chronic inflammation (Chatzinikolaou et al., 2014). Cell culture work with human cells suggests that an increase in oxidative stress causes the activation of nuclear factor-kappa B (NF-κB), a transcription factor that upregulates the synthesis of proinflammatory cytokines, including IL-6, thus linking higher IL-6 levels with chronic inflammation and aging mechanistically (Sarkar and Fisher, 2006). The advantage of this phenotype is that DNA-damage driven inflammation enables angiogenesis, which promotes the invasion and metastasis of tumor cells (Chatzinikolaou et al., 2014). In contrast, a continued accumulation of cellular damage, leading to increases in proinflammatory pathways over time, leads to chronic inflammation, tissue malfunction and degeneration in old age (Chatzinikolaou et al., 2014). Considering that small breed dogs are usually longer lived compared with larger breeds, and that we expect more of an increase in IL-6 for small dogs compared with large dogs, it seems that the mechanism or pathway for increases in IL-6 is not related to stimulation of proinflammatory responses due to DNA oxidative damage accumulation. Alternatively, if inflammation is a contributor to somatic damage associated with aging, the lower IL-6 in small dogs might facilitate a longer lifespan.

Cytokines produced by fibroblasts support the growth, differentiation and activation of inflammatory cells (Vancheri et al., 1991), suggesting that they are important in immune defenses. The lack of metabolic dysregulation or stimulation in conjunction with the observed increase in IL-6 is intriguing. When fibroblasts from clinically normal duodenum tissues of humans were stimulated with LPS, those cells synthesized new IL-6, IL-8 and granulocyte-macrophage colony-stimulating factor (Pang et al., 1994), rather than releasing stored cytokines. If the fibroblasts used in this study also synthesize new cytokines, it follows that the cells must be downregulating other processes to support the production of IL-6 without also increasing ATP production.

Here, we have demonstrated that most of cellular metabolism in primary fibroblasts does not change significantly with age, size or LPS treatment. However, we did see differences in IL-6 concentration in young animals whereby cells from small breed dogs had higher IL-6 concentrations than those from large breed dogs. We also found that IL-6 concentration increased more dramatically with aging after LPS treatment in small breed compared with large breed dogs. This implies that the patterns of inflammaging in dogs may be distinct and different from other mammals commonly studied.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.G.J.; Methodology: A.G.J., C.J.D., W.C.; Validation: A.G.J., W.C.; Formal analysis: S.L., W.C.; Investigation: A.G.J.; Resources: A.G.J., C.J.D., S.L., W.C.; Data curation: S.L., W.C.; Writing - original draft: A.G.J.; Writing - review & editing: C.J.D., S.L., W.C.; Supervision: W.C.; Project administration: A.G.J., W.C.; Funding acquisition: A.G.J., C.J.D.

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Data availability
Full statistical models and data analyses are available on Figshare at: https://figshare.com/s/ee6a5f273a542b703857.

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