To the editor:

Fetal calf serum contains heat-stable nucleases that degrade neutrophil extracellular traps

Extracellular traps (ETs) have been recently discovered as a new paradigm in the innate immune function of leukocytes.[1] Released by neutrophils,[1] mast cells,[2] or eosinophils[3] in response to various infectious or proinflammatory stimuli, ETs represent complexes of nuclear or mitochondrial DNA together with proteins such as histones, cell-specific proteases (eg, myeloperoxidase) and antimicrobial peptides (eg, cathelicidins). DNA is the major structural component of ETs, since treatment with nucleases leads to their dissolution. ETs exhibit strong antimicrobial and/or immunomodulating properties and play an important role in vivo during several infectious and noninfectious diseases.[4]

Interestingly, formation of ETs has consistently been found to be inhibited in a concentration-dependent fashion by serum,[2,5] as we corroborate with neutrophil ETs (NETs) in Figure 1A. Because reactive oxygen species are an essential trigger in the formation of ETs, it was previously hypothesized that fetal calf serum (FCS) impairs the formation of ETs based on its antioxidant activity. Here, we demonstrate that FCS contains heat-stable nucleases that can degrade ETs.

In general, DNase I is regarded as the major serum nuclease. A recent report by Napirei et al[6] showed an additional DNase I–like nuclease to be present in serum; however, detailed information about the heat stability of these nucleases is currently unavailable. As shown in Figure 1B, medium supplemented with 10% FCS, that was inactivated in the standard fashion at 56°C for 30 minutes, showed the ability to degrade calf thymus DNA, indicating the presence of heat-stable nucleases. Similarly, Segal et al[7] reported that serum contains heat-stable nucleases that have the ability to degrade oligodeoxynucleotides (ODNs). We observed that heat inactivation of at least 70°C for 30 minutes was required to prevent FCS degradation of DNA (Figure 1B). Similar nuclease activity was found in 56°C-treated, but not 70°C-treated, mouse serum, human serum, and human plasma (Figure 1B).

Accordingly, we tested whether FCS can degrade ETs. Human blood–derived neutrophils were stimulated with 25nM phorbol-12-myristate-13-acetate (PMA) for 2 hours to release NETs and 10% FCS (heat-inactivated at 56°C or 70°C) was then added to the medium for an additional hour. The presence of NETs was visualized by confocal immunofluorescence microscopy. As shown in Figure 1C, addition of 10% FCS, inactivated in the standard fashion at 56°C for 30 minutes, resulted in a distinct degradation of NETs, whereas FCS inactivated at 70°C did not. This finding was corroborated in a NET quantification assay (Figure 1A). Fixation with 4% paraformaldehyde, which is often used as a blocking agent before immunostainings, did not prevent degradation of ETs by 56°C-inactivated FCS (Figure 1C).

The presence of heat-stable nucleases in serum might explain why visualization of ETs remains a challenge. It may simply be that existing cell-culture protocols (which normally contain 5%-10% FCS) hamper their discovery. Interestingly, nuclease activity is also present in aged solution of bovine plasma albumin Fraction V,[8] which is widely used during in culture experiments as an alternative to FCS. Based on this knowledge, investigators should reconsider which medium and supplements are used to perform in vitro experiments studying the role of ETs. Furthermore, it opens the question of whether formation ET formation by additional leukocyte lineages, such as monocyte/macrophages, or in response to additional environmental stimuli, might also be detected under improved cell-culture conditions using nuclease-free medium supplements.

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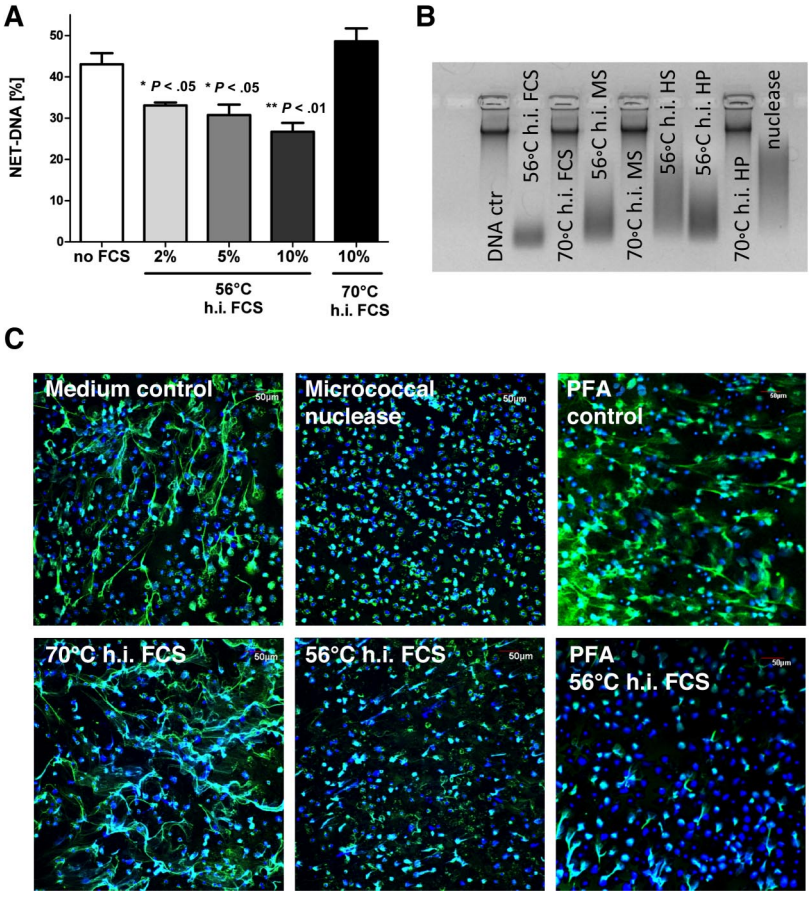

Figure 1. Degradation of neutrophil extracellular traps (NETs) by fetal calf serum (FCS). (A) Quantification of percentage of NET-DNA (using Picogreen [Invitrogen] as previously described[5]) released by human blood–derived neutrophils (isolated using PolymorphPrep system [Axis-Shield] as recommended by the manufacturer) after stimulation with 25nM PMA for 3 hours at 37°C and 5% CO_2 in the presence of different concentration with FCS. Data are mean and SEM of 3 independent experiments. Significant differences analyzed by unpaired *t* test. (B) Activity of RPMI medium (Invitrogen) containing 10% FCS (Invitrogen), mouse serum (MS), human serum (HS), or human plasma (HP) in degrading 150 μg/mL calf thymus DNA (Sigma) as determined by agarose gel electrophoresis. Serum or plasma was heat-inactivated at 56°C or 70°C for 30 minutes before experiments. Note that medium containing 56°C heat-inactivated serum or plasma showed degradation of DNA similar to micrococcal nuclease used as a positive control. In contrast, heat inactivation of serum or plasma at 70°C completely abolished this nuclease activity. (C) Confocal immunofluorescence microscopy to visualize degradation of NETs by FCS. Human blood–derived neutrophils were stimulated with 25nM PMA for 2 hours at 37°C and 5% CO_2 in serum-free RPMI to release NETs. Then, 10% FCS heat-inactivated at either 56°C or 70°C was added to the medium for an additional hour. As control, NETs were degraded by adding 500 mU/mL micrococcal nuclease (Worthington Biochemical Corporation) to the medium. NETs were visualized by immunofluorescence microscopy using a rabbit anti myeloperoxidase-antibody (1:300; 1 hour at room temperature; Dako), followed by a secondary Alexa 488–labeled goat anti–rabbit antibody (1:500; 1 hour at room temperature; Invitrogen); samples were embedded in ProlongGold+Dapi (Invitrogen) to counterstain nucleus and extracellular DNA in blue. Mounted samples were examined using an inverted confocal laser-scanning 2-photon microscope Olympus Fluoview FV1000 with Fluoview TM Spectral Scanning technology (Olympus) and a 20×/0.75 UPlanSApo Olympus objective. Note that addition of 56°C heat-inactivated FCS to the cells, in contrast to FCS heat-inactivated at 70°C, resulted in degradation of NETs. Fixation of cells with 4% paraformaldehyde (PFA) for 15 minutes at room temperature did not prevent NET degradation by 56°C heat-inactivated FCS.