Temperature sensitivity of bat antibodies links metabolic state with antigen-recognition diversity

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Abstract

Immune system in bats features multiple unique properties that result in overall dampened inflammatory responses and increased tissue protection, explaining their tolerance to viral infections and unusually long lifespan. As heterothermic animals, bats exhibit large variations in body temperature on a daily and seasonal basis. In the present study, we demonstrated that temperature fluctuations corresponding to different physiological states in bats exert a dramatic impact on bat antibody repertoires. At temperatures typical for elevated metabolic activity during flight, IgG antibodies from *Myotis myotis* (Borkhausen, 1797) and *Nyctalus noctula* (Schreber, 1774) largely broaden their antigen binding diversity and affinity, recognizing both pathogen-derived and autoantigens. On the contrary, at temperatures reflecting inactive physiological states (torpor, hibernation), the breadth of immune diversity is substantially reduced. Importantly, temperature-dependent diversification of antibody specificities results in preferential recognition of damaged but not alive endothelial cells. This indicats that metabolism might influence not only pathogen recognition function but also anti-inflammatory functions of bat antibodies. Our mechanistic studies revealed particular molecular features of bat IgG, such as low thermodynamic stability and increased hydrophobicity, that can explain their distinctive behavior. This study reveals another facet of the enigmatic immune system of bats, extends our understanding of disease tolerance, highlights the link between metabolism and immunity, and might have important repercussions for human health.

Keywords:

Antibodies; Bats; Immune diversity; Bat immunology; Antigen binding; Body temperature; Metabolism; Disease tolerance; Immune homeostasis; Oxidative stress.

Introduction

Bats (order *Chiroptera*) represent the second largest order of mammals, after rodents, numbering more than 1400 species. They are the only mammals capable of powered flight and possess several exceptional physiological characteristics such as long lifespan, low incidence of age-related diseases, and elevated tolerance to viral infections [1]. Different bat species have been reported to harbor a particularly high diversity of viruses [2-7], some of which can cause severe diseases in humans [8]. Yet, bats rarely succumb to immunopathology, thanks to some unique evolutionary adaptations of essential immune pathways [1,8-10]. For instance, it has been shown that bats feature a particular genetic and functional organization of the IFN system that can manifest in constitutive tissue expression of IFNs, differential expression of IFN-stimulated genes, and altered kinetics of IFN responses [11,12]. Additionally, in contrast to primates and rodents, many bat species demonstrate impairments in the detection and response to DAMPs, such as cytosolic DNA. This includes blunted activity of the NLRP3 inflammasome due to the presence of a splice variant with a lower ability for ASC speck formation and activation of IL-1β [13]. Other studies have shown reduced proteolytic activity of caspase-1 or reduced cleavage susceptibility of IL-1β by caspase-1 in bats [14] as well as a complete deficiency of certain members of PYHIN gene family, which are involved in DNA sensing and activation of the inflammasome [15]. A number of bat species carry a conservative mutation reducing the activity of STING [16], a central DNA sensing protein orchestrating type I IFN responses. Regarding immune receptors, genomic studies of the Egyptian fruit bat *Rousettus aegyptiacus* (Geoffroy, 1810) have indicated predominant expression of NK receptors with inhibitory activity, Fc-γ receptors with reduced affinity for IgG, and MHC I molecules able to present higher diversity of antigenic peptides [17,18].

In addition to the genetic features of their immune components, physiological peculiarities of bats may also play a significant role in tolerance to viral infections. In particular, body

temperature in bats can significantly fluctuate on a seasonal and quotidian basis, especially in temperate regions. During the metabolically demanding flight, it is usually around 40 °C, reaching up to 42°C in some species [19], however, it can drop to ≈20 °C during shallow torpor [20] and even below <10 °C during hibernation [21,22]. As a consequence, both high and low body temperatures have been proposed as a mechanism to control infection by providing suboptimal conditions for pathogen replication. In detail, the rise in temperature during flight creates a similar physiological condition as the febrile response in other mammals (so called "flight-as-fever" hypothesis), which is recognized as an evolutionary ancient immune defense mechanism [19,23]. On the other hand, even short torpor bouts have been shown to reduce viral growth rate sufficiently to control infection [20]. Yet, it is barely known how temperature fluctuations affect the innate and adaptive immune compartments of bats and how this can contribute to increased pathogen tolerance.

In the present study, we aimed to fill this gap by rationally planning experiments based on the specific life history traits and physiological characteristics of bats. In particular, we concentrated on antibodies as the major component of humoral immune response with central roles in adaptive immune defense against pathogens and maintenance of immune homeostasis [24]. Our data revealed an unconventional mechanism for diversification of immune specificity of IgG antibodies driven by temperature in two bat species, the Greater moused-eared bat (*Myotis myotis*) and the Common noctule (*Nyctalus noctula*). Additionally, we described unique physicochemical features of bat antibodies that might explain temperature sensitivity. Finally, we identified potential immune defense, homeostatic and anti-inflammatory functions of this phenomenon that contribute to better understanding of elevated disease tolerance in bats.

Results

Temperature modulates the antigen-binding function of bat IgG antibodies

As a consequence of their specific diurnal and seasonal cycles, bats experience large fluctuations in body temperature, and so does their immune system [10,19,21,22,25-29]. This might be particularly relevant to antibodies as they rely on molecular recognition to provide antigen specificity, a process that is strongly affected by a variety of physicochemical factors. Thus, we investigated antigen-binding properties of bat antibodies at three different temperatures: 4, 22 and 42 °C, reflecting physiological states of hibernation, shallow torpor, and active flight, respectively. To this end, we purified total IgG from sera of two bat species, *M. myotis*and *N. noctula*, as well as of healthy humans to serve as a reference point. Then, we analyzed antibody binding as a function of temperature to a panel of 22 unrelated mammalian and pathogen-derived proteins and LPS using high-throughput ELISA (Fig. 1a and Suppl. Fig. 1). The obtained results revealed only negligible reactivity of polyclonal IgG from both bat species to studied antigens at 4 and 22 °C. However, the same IgG samples demonstrated statistically significant broadening of their antigen recognition potential at 42 °C (Fig. 1a and Suppl. Fig. 1). At high temperature, bat antibodies augmented their reactivity to almost all antigens in the panel, yet four proteins were identified as preferentially recognized outliers: NS1 protein of TBEV, rabies virus glycoprotein, measles virus hemagglutinin, and thyroglobulin (Fig. 1a and Suppl. Fig. 1b). In contrast, immune specificity of human IgG, both pooled and from individual donors, was not significantly affected by temperature (Fig. 1a and Suppl. Fig. 1b), whereby it demonstrated significant (and independent from temperature within the range of 4 – 42 °C) binding to LysM (*E. faecalis*), LPS (*E. coli*), SARS-CoV-2 S protein and influenza virus haemagglutinin (Fig. 1a and Suppl. Fig. 1a). Notably, at 42 °C, human IgG demonstrated a tendency for reduced binding to LPS.

Next, we applied surface plasmon resonance to quantify the effect of temperature on antigen-binding properties of bat antibodies. We assessed antibody binding to the two antigens recognized with the highest intensity in the previous ELISA experiment: NS1 protein of TBEV and rabies glycoprotein (Fig. 1b). The real-time kinetic analysis demonstrated that binding of IgG from *M. myotis* to both proteins progressively increased as a function of temperature. At temperatures within the range of 4-16 °C, binding was barely detectable even at the highest antibody concentrations. In contrast, at the highest temperature achievable with the instrument (40 °C), interaction of bat IgG was considerable and subjectable to global kinetic analysis (Suppl. Fig. 2). These showed that bat IgG bound to NS1 protein and rabies virus glycoprotein with physiologically relevant values of apparent binding affinity (K_D values of 26 and 189 nM, respectively; Fig. 1b and Suppl. Fig. 2).

To understand whether reactivity of bat antibodies is still controlled by temperature in a complex biological system, we also performed ELISA experiments using whole sera (Fig. 1c). Again, binding of bat IgG to NS1 of TBEV and rabies glycoprotein profoundly increased with temperature, in contrast to binding of human IgG to SARS-CoV-2 S protein and influenza virus haemagglutinin. (Fig. 1c). These results were in complete agreement with our previous findings obtained with purified IgG. However, we noted that reactivity of bat IgG to the blocking agent was elevated in complete sera as compared with purified IgG (Suppl. Fig. 3). This could be explained by an interference effect of other proteins and/or detection of binding of immunoglobulins other than IgG present in complete sera.

To further confirm that temperature sensitivity of bat antibodies is a species-specific and not an individual trait, we used 12 additional serum pools from *M. myotis* to test IgG binding to NS1 of TBEV. As expected, at a temperature typical for high metabolic rate (42 °C), there was a significant increase in IgG binding to the antigen. Importantly, the phenomenon was observed

in sera collected both before and after hibernation, a period associated with a significant suppression of immune functions and a decrease in antibody production [30] (Suppl. Fig. 4).

Finally, using immunofluorescence, we elucidated reactivity of bat IgG to HEp-2 cells, which is traditionally used to detect the presence of autoantibodies. Our results indicated only negligible binding of both human and bat IgG to Hep-2 at 22 °C, with bat antibodies demonstrating higher reactivity (Fig. 1d). However, at 42 °C, bat antibodies markedly increased their binding to different cellular structures, both cytosolic and nuclear, a typical characteristic of autoantibodies (Fig. 1d and Suppl. Fig 5); this was not observed with human IgG.

Collectively, results from this part show that bat IgG (purified or in whole sera) considerably increase their antigen-binding reactivity towards pathogen-derived and mammalian antigens at temperatures typical for high metabolic rate (observed during flight). On the contrary, temperature does not change reactivity of human IgG.

Temperature broadens diversity of bat antibody antigen specificities

In order to obtain more integrative insights about the effect of temperature on bat (and human) antibody repertoires, we applied protein array technology. Human proteome arrays HuProt™ display >17 000, different proteins, thus allowing the study of immune reactivity to enormous diversity of targets with different sequences and structural features. In accordance with the previous assays, our pool of purified IgG from *M. myotis* manifested only negligible binding to protein targets at 22 °C. However, when the same pool was probed at 42 °C we observed a marked broadening in the repertoire of recognized proteins (Fig. 2a). At elevated temperatures, bat IgG recognized a significant fraction of tested proteins (>2400 or ≈ 14 % of all proteins) with a high binding intensity (MFI > 1000), whereby only 19 proteins were recognized with a high binding intensity at 22 °C. In contrast, temperature affected binding diversity of human

IgG at a considerably lower extend (Fig. 2a). At 42 °C, human antibodies recognized only 134 proteins with a high binding intensity – a limited increase in antigen-binding diversity as compared to 22 °C where human antibodies interacted strongly only with 8 proteins (Fig. 2a). The considerable difference in the diversity of immune recognition specificities of bat and human is obvious when the reactivity of antibodies from the two species was plotted (Fig. 2b). Thus, at 22 °C bat and human antibodies displayed similar pattern for immune reactivity. In contrast at 42 °C there is considerable asymmetry in the protein binding landscape (Fig. 2b). Finally, to determine the interactions of antibodies with high specificities i.e., the ones that show statistically higher protein binding than average reactivity towards all proteins, we evaluated the Z-scores. The plots of Z-scores indicated that at high temperature a larger set of proteins are recognized with high specificity by bat IgG as compared to human IgG (Fig. 2c). These data indicated that bat antibodies can significantly broaden their antigen binding diversity at temperatures typical for active metabolic state. On the contrary, antigen binding diversity of human antibody repertoire is much less affected by temperature variation.

Particular physicochemical qualities of bat antibodies can contribute to their temperature sensitivity

To get an insight into the molecular mechanism of the noncanonical diversification of immune specificities in bat antibodies, we studied their molecular properties. First, we estimated the thermodynamic stability of IgG molecules using a thermal shift assay. It revealed that the unfolding temperature (or melting point Tm) of *M. myotis* IgG was significantly lower as compared to the Tm of human IgG (Fig. 3a), indicating lower thermodynamic stability. This was also confirmed using a second bat species, *Myotis capaccinii*, (Suppl. Fig. 6). Additionally, we estimated the overall hydrophobicity of bat IgG using fluorescence spectroscopy with a specific molecular probe, 1-anilinonaphthalene-8-sulfonic acid (ANS, Fig. 3b). The experiment

demonstrated that bat IgG was characterized by a substantially higher hydrophobicity as compared to human IgG.

Together, these results reveled some distinctive physicochemical features that can determine particular temperature sensitivity of bat IgG.

Higher prevalence of polyreactive antibodies in bat immune repertoires might contribute to unconventional diversification of immune specificity as a function of temperature

In studies with murine monoclonal IgG and human Fab fragments, it was demonstrated that polyreactive antibodies (or Fab), contrary to monoreactive ones, bind to an increased number of antigens at high temperatures [31,32]. To adapt these findings to the context of our study, we analyzed the reactivity of the widely used as control for human monoclonal polyreactive antibody ED38 as well as of two monoreactive antibodies: VRC01 (specific for gp120 of HIV-1) and S309 (binding to spikes of both SARS-CoV-1 and SARS-CoV-2) (Fig. 1a). As previously, we performed an ELISA using the abovementioned panel of 22 antigens at 4, 22 and 42 °C. The obtained data demonstrated that the polyreactive antibody ED38 significantly increased its binding as a function of temperature to most of the model antigens (Fig 4a and Suppl. Fig. 7). In contrast, the two monoreactive antibodies did not broaden their antigen reactivity pattern. Notably, even an opposite effect was observed in VRC1, where its binding to gp120 slightly decreased at 42 °C (Fig 4a). To test whether temperature sensitive antibodies in bats are also polyreactive, we applied a molecular probe, dinitrophenol (DNP) conjugated to BSA, that has been proven reliable for the estimation of overall levels of polyreactive antibodies in antibody repertoires [33-35]. Thus, we compared binding of human and bat (*M. myotis* and *N. noctula*) purified IgG pools to immobilized DNP. Our results indicated a considerably higher reactivity (2-3-folds higher) of antibodies from both bat species to the

probe as compared to antibodies isolated from two serum pools from healthy humans (Fig. 4b). We also demonstrated that DNP well distinguished polyreactive from monoreactive antibodies at 22 °C (Fig. 4c). Overall, these data suggested that bat antibody repertoires contain considerably higher levels of polyreactive antibodies, which might at least partly explain the diversification of their antigen-binding reactivity at high temperatures.

Elevated temperature preferentially triggers the reactivity of bat antibodies to dead cells

Next, to provide understanding of the physiological role of temperature-sensitive IgG, we investigated the capacity of bat and human antibodies to opsonize endothelial cells. The marked intensification of bat metabolic rate during flight is associated not only with increased body temperature but also with the release of ROS, that can result in cell death and liberation of various endogenous molecules with pro-inflammatory activity [1]. The temperature-dependent augmentation of antibody reactivity to mammalian proteins can represent a mechanism for clearance of damaged or dead cells, which have strong pro-inflammatory potential. To test this hypothesis, we modeled induction of cell death by oxidative stress through the exposure of endothelial cells HMEC-1 to hydrogen peroxide. Gating strategy for identification of necrotic endothelial cells is presented in Figure 5a. Labeling cells with annexin V and propidium iodide clearly indicated that exposure to the pro-oxidant triggered endothelial cell dead. To assess the impact of temperature on reactivity of bat and human IgG to cells with different viability, we incubated antibodies at 22 and 42 °C and tested their capacity to opsonize alive and dead cells by flow cytometry (Fig. 5b). Both human and bat IgG demonstrated low reactivity to alive HMEC-1 cells, which was negligibly changed when incubation was performed at high temperature (Fig. 5b). Antibody reactivity to dead cells at 22 °C was also low for both human and bat samples, yet, bat antibodies demonstrated a slight but statistically significant increase in binding ($p < 0.05$, Wilcoxon test) (Fig. 5c). In contrast, at 42 °C, binding of bat IgG to dead

cells was markedly augmented (Fig. 5b and 5c), while reactivity of human IgG was not modified by the increase in temperature.

Together, these data indicate that bat antibodies acquire preferential reactivity to dead endothelial cells at elevated temperatures. This would result in their efficient opsonization and contribute to their rapid clearance during a metapolitically active physiological state, thus exerting an important anti-inflammatory activity.

Discussion

The present study draws a direct connection between specific life history traits and physiological characteristics of bats and the function of their immune compartments. In particular, we demonstrated that temperature fluctuations, accompanying daily and seasonal cycles of bats, can regulate the extent of antigen binding strength and diversity in their antibody repertoires. By elucidating the reactivity of IgG antibodies from two temperate bat species, the Greater mouse-eared bat (*Myotis myotis*) and the Common noctule (*Nyctalus noctula)*, we found that elevated temperatures (40-42 °C), typical for the active metabolic state during flight, significantly broadened their spectrum of recognized antigens. On the other hand, at temperatures representing inactive metabolic states (shallow torpor, 22 °C or hibernation, 4 °C) diversity of antigen binding specificities of bat antibodies substantially collapsed. The same temperature changes did not modulate binding diversity of human antibodies. Importantly, these results are derived from several independent experiments with multiple serum pools, indicating that antibody temperature-sensitivity is a trait typical for at least two, but most probably more, bat species. Furthermore, the application of protein microarrays (containing >17000 proteins) in the study of bat immune system indicates that the observed phenomenon is not dependent on particular antigens and might have a broad physiological relevance.

Importantly, a previous study demonstrated that interactions of murine polyreactive antibodies or human polyreactive Fab fragments were exclusively augmented at high temperatures in contrast to those of monoreactive antibodies or Fab fragments [31,32], an effect that we confirmed with human IgGs. Thus, we hypothesize that elevated frequencies of polyreactive antibodies are likely contributing to temperature-sensitivity and antibody repertoire diversification in bats. Yet, we do not exclude implication of other mechanisms, especially in the cases of antigens recognized with high binding affinities (NS1 of TBEV and rabies virus glycoprotein), which are atypical for polyreactive antibodies. Accordingly, our mechanistic studies of bat IgGs demonstrated that they are characterized by some unusual physicochemical features, such as significantly lower thermodynamic stability and higher surface hydrophobicity as compared to human IgGs. The thermodynamic stability of proteins is determined by the complexity of networks of noncovalent interactions that maintain the protein fold. Thus, the polypeptide chains of less stable proteins are usually less tightly packed and consequently these proteins have increased structural dynamics [36-38]. Based on these notions decreased thermodynamic stability of bat antibodies can signify thar they are characterized by enhanced conformational dynamics. The latter can be especially increased as a function of temperature. Higher flexibility of antigen-binding site can allow increased binding adaptability and recognition of a large number of antigens [39-41] or targeting of epitopes that are difficult to be recognized by conventional antibodies [42]. Another untypical physiochemical quality of bat antibodies determined in this study is their high surface hydrophobicity. This feature may also explain the temperature effects as it was found that molecular interactions that are mainly driven by hydrophobic interaction increase their strength as function of temperature [43,44] – a fact in full concordance with our data.

A key aspect of this study is that it reveals an important physiological mechanism that links metabolism and immunity and may contribute to understanding of increased disease tolerance in bats. Such conclusions are in full concordance with previous research suggesting the evolution of powered flight as a main driver of immune system evolution in bats [10,45,46]. Indeed, flight in bats is associated with a substantial increase in metabolic rate (>30 folds), rise in body temperature, and intensification of oxidative processes that can lead to cell damage and the release of various DAMP molecules such as DNA, misfolded proteins, oxidized phospholipids, ATP, etc [1,25,47,48]. As an adaptation to such metabolic stress, the activity of innate pathways sensing these molecules is reduced in bats, thus protecting them from perpetual inflammation [9,10,45] – a phenomenon that has been also associated with bat tolerance to viral infections [10].

The unconventional mechanism for control of antigen binding diversity, proposed here, could further contribute to this tolerance and may as well have other important biological repercussions. Immunogenetic studies have demonstrated that the diversity of functional gene segments encoding variable immunoglobulin regions in bats is similar to that of humans and mice [18,49-51]. Thus, the potential repertoire of bat antibody specificities seems sufficiently large to cope with high versatility of pathogens, without the need of any extraordinary diversification strategies. Yet, immune reactivity of bat antibodies that correlates to (and is controlled by) the state of metabolic activity might be biologically meaningful by providing a physiological adaptation for optimizing immune response timing. For instance, concealing antibody reactivity at low temperatures might be an additional strategy to economize energy and resources during phases of inactivity and suppressed metabolism, i.e., daily torpor or seasonal hibernation [52,53]. As pathogen replication is severely inhibited at low temperatures typical for torpor, some immune components may not be necessary to control infection during this period. This is particularly true for adaptive immune responses that are known to be one of the most energy-demanding processes in mammals [54,55]. On the other hand, substantial antibody

diversification observed at high temperatures may provide bats with an instant protection upon exiting torpor and returning to body temperatures that are favorable for pathogen replication. Such acting at the first line of immune defense is typical for polyreactive antibodies that allow the immune system to rapidly cover a practically infinite antigenic space, albeit with lower affinity. Interestingly however, bat IgG also shows a temperature-dependent rise in binding strength to specific antigens as exemplified by our ELISA and real-time kinetic experiments with NS1 protein of TBEV and rabies virus glycoprotein. Both have been previously detected in bat species used in our study [56-59], therefore, the apparent binding affinity of bat IgG to them at high temperature may indicate shaping of immune repertoire as a result of coevolution of bats with these or other related viruses.

Another important aspect of bat antibody temperature-sensitivity is the potential to control inflammation induced by the accumulation of dead cells and the release of DAMPs during flight. This is shown by our experiments where bat IgG preferentially bound and opsonized dead endothelial (and epithelial Hep2) cells but speared living cells at elevated temperatures. Thus, increase in antibody reactivity during active metabolic states probably contributes to the clearance of apoptotic cells and damaged macromolecules *in vivo*, providing a new anti-inflammatory mechanism in addition to the ones already described in bats. Of note, studies in mouse models and humans proved the important role of natural polyreactive antibodies in maintenance of immune homeostasis by clearance of damaged or dead cell [60-64]. This is in accordance with our data showing that bat antibody repertoires contain a significant fraction of polyreactive antibodies.

Taken together, the results from this study uncover new particularities of bat immune system and provide another explanation of bat tolerance to inflammation and disease. These findings also depict an alternative mechanism for diversification of immune specificity of antibodies in general and highlight the link between metabolism and immunity. Further elucidating the

mechanism of temperature sensitivity of bat antibodies will extend our understanding of immune system evolution in mammals and can lead to practical discoveries with important repercussions for human health.

Methods

Bat capture and blood sampling

For this study, we used samples from 3 different bat species: Greater moused-eared bat (*Myotis myotis*), Long-fingered bat (*Myotis capaccinii*), and Common noctule (*Nyctalus noctula*). Moused-eared bats were caught at Ivanova voda cave, situated in Western Rhodopes mountain, Bulgaria (N41.894, E24.880), in April 2021 (n = 40), October 2021 (n = 45), and May 2022 (n = 29). Long-fingered bats were also caught at Ivanova voda in April 2021 (n = 3) and May 2022 (n = 20). Noctule bats were caught at Devetashkata cave, situated in North Central Bulgaria (N43.233, E24.885), in June 2022 (n = 18). Bats were caught at cave entrances at sunset using a harptrap, then they were kept in individual cotton bags until processing. Their sex, size, weight, and approximate age were noted. For species identification, we followed Dietz and Von Helversen (2004). The research was carried out under a permit by the Bulgarian Biodiversity Act (No 830/19.09.2020 and No 927/04.04.2022).

Blood samples were taken from the uropatagium vein by puncturing it with a sterile disposable insulin needle and collecting the few drops of leaked blood (most often below 100 µL) using a sterile disposable hematocrit capillary or a pipette with sterile disposable tips. Blood sampling of this kind is not supposed to affect survival rates of bats [65]. Blood serum was immediately separated from plasma by centrifugation for 15 to 30 mins at 2000 rpm. Separated sera were then frozen at -20 °C. Each sampled bat was given water and mealworms (*Tenebrio*) to compensate for blood loss or considered minimizing handling time to reduce stress in the

animals. All researchers used personal protective equipment (masks, gloves) and non-disposable instruments and working surfaces were disinfected between each animal. To obtain enough material for experiments, pools sera from 3 to 8 individuals was pooled in the lab or directly in the field. Additionally, in the study we used fresh AB serum samples from nine anonymised healthy human donors, obtained under the ethical convention between INSERM with Etablissement Français du Sang # 18/EFS/033.

IgG purification

Total IgG from both human and bat sera was purified using Melon^{TM} Gel IgG Spin Purification Kit (Thermo Fischer Scientific, Waltham, MA) according to the manufacturer's protocol, with the sole exception that centrifugation was performed for 30 sec instead of 10 sec. Concentration of purified IgG was measured by a Nanodrop spectrophotometer (Thermo Fischer Scientific) and its purity was confirmed by SDS-PAGE performed in reducing and non-reducing conditions.

ELISA –temperature dependence of antigen binding of bat and human IgG

Thermo Scientific™ Nunc 384-Well Clear Polystyrene Plates (Thermo Fischer Scientific) were coated overnight at 4 °C (or alternatively for 2 hours at room temperature, 22-24 °C) with the following molecules: human Factor VIII (Helixate® FS, CSL Behring, King of Prussia, PA); human Factor IX; human von Willebrand factor (both from LFB, Les Ulis, France); human insulin, human hemopexin, human hemoglobin, porcine thyroglobulin, bovine histone III, equine cytochrome C, lipopolysaccharide from *Escherichia coli* O55:B5 (all from Sigma-Aldrich, St. Louis, MO); LysM protein (from *Enterococcus faecalis*, kindly provided by Dr. Stephane Mesnage, University of Sheffield, UK); Asp f1 protein (from *Aspergillus fumigatus*,

kindly provided by Dr. Vishu Kumar Aimanianda, Institute Pasteur, Paris); spike protein of SARS-CoV-2 (S protein, Wuhan variant), receptor-binding domain (RBD, SARS-CoV-2 Wuhan variant), human ACE2 (the three proteins were kindly provided by Dr. Hugo Mouquet, Institute Pasteur, Paris, France); gp120 from HIV-1 (strain CN54, kindly provided by NIH AIDS Reagents Program); rabies virus glycoprotein, Ebola Zaire virus glycoprotein, Nipah virus glycoprotein, Hendra virus glycoprotein, NS1 protein of tick-born encephalitis virus (TBEV), measles virus mosaic hemagglutinin (viral proteins were obtained from Immune Technology, New York, NY). Plates were incubated with proteins usually diluted to 5 μg/ml in PBS; insulin and LPS were diluted at 10 μg/ml. After the coating step, the microtiter plates were blocked by incubation for 2 hours with PBS containig 0.25 % Tween 20. Alternatively, other blocking agents were applied, 1 % solution of bovine serum albumin (Sigma-Aldrich) in PBS or 1 × solution of synthetic block (Thermo Fisher Scientific) in PBS. Following the blocking step, the plates were incubated with: IgG purified from pooled sera of two bat species, *M. myotis* and *N.* noctule; IgG purified from sera of healthy humans; human monoclonal IgG1 antibodies ED38 (highly polyreactive antibody), VRC01 (specific for HIV-1 gp120), and S309 (specific for RBD of SARS-CoV-2), serving as controls. Polyclonal IgG from both human and bats was typically diluted to 0.07 mg/ml (or wherever indicated to 0.1 mg/ml) in PBS containing 0.05 % Tween 20 (PBS-T). Monoclonal IgG antibodies were diluted to concentrations of 0.01 and 0.07 mg/ml in PBS-T. In a particular experimental setting, reactivity of IgG in whole sera from *M. myotis* or humans were tested. To this end, sera were diluted 200 × in PBS-T. Purified polyclonal IgG and diluted sera from bats and humans, or monoclonal antibodies were incubated for 2 hours with immobilized antigens in parallel at three different temperatures: 4 °C, 22 °C, and 42 °C (one plate per temperature). After, the microtiter plates were washed 5 × with PBS-T and then incubated for 1 hour at room temperature with 2 μg/ml of biotinylated recombinant protein G (Pierce™, Thermo Fisher Scientific) for recognition of

both bat and human IgG antibodies. Following thorough washing, streptavidin conjugated with HRP (Invitrogen, Thermo Fisher Scientific) diluted 100 × was added and incubated for 30 min at room temperature. Following thorough washing with T-PBS, reactivity of human and bat antibodies was revealed by adding peroxidase substrate, *o*-phenylenediamine dihydrochloride (Sigma-Adrich). After the addition of 2M HCl to all wells to terminate the enzymatic reaction, absorbance at 492 nm was measured with a microplate reader (Infinite 200 Pro, Tecan, Männedorf, Switzerland).

In another experimental setting, NS1 protein of tick-born encephalitis virus (TBEV), rabies virus glycoprotein, influenza hemagglutinin (HA(ΔTM) (A/California/04/2009, Immune Technology) and SARS-CoV-2 S protein, were immobilized at 2 μg/ml on 96-well polystyrene microtiter plates NUNC MaxiSorp™ (Thermo Fisher Scientific). Following blocking wit 1 × synthetic block in PBS, whole sera from human and *M. myotis* was serially diluted (starting from 60 × to 1920 ×) in PBS-T and incubated for 2 hours at 4 °C, 22 °C, 42 °C with immobilized antigens (human serum with SARS-CoV-2 S protein and influenza A hemagglutinin; bat sera with TBEV NS1 and rabies virus glycoprotein). Succeeding steps of the experiment are identical as described above.

ELISA –assessment of polyreactivity of IgG antibodies

To assess the level of polyreactive antibodies in polyclonal bat and human IgG samples, we used an established assay based on the analyses of antibody reactivity to nitroaren hapten [35]. Ninety-six well polystyrene plates NUNC MaxiSorp™ or NUNC 384-Well Clear Polystyrene (Thermo Fisher Scientific) were coated with 2,4-dinitrophenyl conjugated to BSA (DNP-BSA, Sigma-Aldrich), diluted to 10 μg/ml in PBS and incubated for 2 hours at room temperature (22-24 °C). As a control, unconjugated BSA was coated under identical conditions. The plates

were blocked by saturation with 0.25 % solution of Tween 20 in PBS for 1 hour at room temperature. For evaluation of antibody reactivity to DNP, IgG purified from human and bat (*M. myotis* and *N. noctula*) pooled sera was diluted to 670 nM (100 μg/ml) in PBS-T, followed by two-fold serial dilution to 10.468 nM (1.56 μg/ml). Monoreactive (VRC01 and S309) and polyreactive (ED38), monoclonal IgG1 antibodies were used as controls. These were first diluted to 400 nM (60 μg/ml) followed by three-fold serial dilutions to 0.0068 nM (0.001 μg/ml) in PBS-T. Polyclonal and monoclonal antibodies were incubated on microtiter plates for 1 hour at room temperature. Following steps were as described above.

Real-time interaction analyses

Interaction of bat IgG with selected viral proteins was evaluated by a surface plasmon resonance-based system – Biacore 2000 (Cytiva, Biacore, Uppsala, Sweden). Recombinant rabies virus glycoprotein and NS1 protein of TBEV were covalently immobilized on CM5 sensor chips (Cytiva, Biacore) using an amine-coupling kit (Cytiva, Biacore) according to the procedure recommended by the manufacturer. In brief, viral proteins were diluted to a final concentration of 10 μg/ml in 5 mM maleic acid, pH 4, and injected over pre-activated sensor surfaces. Carboxyl groups of the carboxymethylated dextran were activated by exposure of the surface for 4 min to a mixture of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and N-hydroxysuccinimide. For inactivation of the activated carboxyl groups thar were not engaged in interactions with the amino groups from the proteins, sensor surfaces were exposed for 4 min to 1M solution of enthanolamine.HCl. Covalent immobilization of viral proteins and all kinetic measurements were performed with HBS-EP (10 mM HEPES pH 7.2; 150 mM NaCl; 3 mM EDTA, and 0.005 % Tween 20) used as running and sample dilution buffer. Prior to real-

time binding analyses, running buffer was always filtered through a 0.22 µm filter and degassed under vacuum.

To measure interactions of bat antibodies with viral proteins as a function of temperature, IgG purified from a serum pool from *M. myotis* was serially diluted in the running buffer in the range 100 – 6.25 µg/ml (670 – 41.875 nM) and injected over the sensor surface. Flow rate during all interaction analyses was set at 30 µl/min. The association and dissociation phases of bat polyclonal IgG binding were monitored for 4 and 5 min, respectively. Between consecutive sample injections, the sensor chip surface was regenerated by a brief exposure (30 sec) to a solution of 2M NaSCN (Sigma-Aldrich). All analyses were performed at the following temperatures: 4, 6, 16, 24, 32, and 40 °C. Real-time binding curves were obtained after subtracting bat IgG binding to a reference surface that lacked immobilized virus proteins. Binding curves obtained at 40 °C allowed reliable evaluation of binding kinetics. To this end, we used BIAevaluation software v. 4.1.1 (Cytiva, Biacore) performing global kinetics analyses applying the Langmuir binding model. The apparent binding kinetics and affinity were evaluated under the assumption that 10 % of all antibodies in the pooled preparation were able to bind viral proteins.

Conjugation of human and bat IgG with fluorochromes

Human and bat antibodies were covalently labelled with fluorochromes via amine-coupling reaction. First, polyclonal IgG purified from pooled human and bat (*M. myotis*) sera was diluted to 2 µM in a solution consisting of 1/3 100 mM carbonate buffer, pH 9, and 2/3 PBS. Amine-reactive Alexa Fluor™ 555 NHS ester (Thermo Fisher Scientific) was added to IgG samples to final concentrations of fluorochrome 40 µM and incubated for 1 hour at room temperature with constant gentle agitation. Additionally, human and bat IgG were labeled with Alexa

Fluor™ 647 NHS ester under identical conditions. Following the conjugation reaction, we separated labeled IgG from unconjugated fluorochromes applying PD-10 columns with Sephadex G-25 resin (Cytiva, Marlborough, MA). Conjugation efficacy and conjugated IgG concentration was measured by absorbance spectroscopy using Agilent Cary 100 UV-vis spectrophotometer (Agilent Technologies, Santa Clara, CA).

Immunofluorescence analyses

To evaluate reactivity of bat (*M. myotis*) and human IgG to HEp2 cells, we used the AESKUSLIDES® ANA-HEp-2 diagnostic kit for detection of autoantibodies recognizing cellular antigens by immunofluorescence (Aesku Diagnostics, Wendelsheim, Germany). We followed the protocol recommended by the manufacturer with certain modifications. Thus, human and bat IgG conjugated to Alexa Fluor™ 555 were diluted to 50 µg/ml in PBS and incubated for 30 min in a moist chamber with Hep-2 cells pre-fixed on glass slides. To evaluate the effect of temperature on IgG binding, slides with identical samples were incubated at 22 °C and 42 °C. As a negative control some cells were incubated with PBS only or with 50 µg/ml of Alexa Fluor™ 555-streptavidin (Invitrogen, Thermo Fisher Scientific). After washing with PBS and applying mounting medium and cover slips, immunofluorescence was read by Zeiss Axio Observer Z1 microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with ApoTome system. Acquired images with magnifications of 40× and 63× were colored digitally in green. The acquisition and data analyses were performed with ZEN software (Carl Zeiss AG). Quantification of mean fluorescence intensity (MFI), reflecting antibody interaction with the cells, was normalized by the number of cells observed in the bright-field.

Protein microarrays

To evaluate the effect of temperature on the immunoreactivity of human and bat (*M. myotis*) polyclonal IgG to a broad repertoire of human proteins (>17 000), we applied HuProt™ v4.0 protein microarrays (CDI Laboratories, Inc., Baltimore, MD). First, microarray slides were blocked by incubation for 2 hours at room temperature with PBS containing 1× synthetic block (Thermo Fisher Scientific) and 0.05 % Tween 20. After washing for 5 min with PBS containing 0.05 % Tween 20 (PBS-T), chips were incubated for 2 hours at 22 °C or 42 °C with Alexa Fluor™ 647-conjugated human and bat IgG (for 22 °C) and Alexa Fluor™ 555-conjugated human and bat IgG (for 42 °C) with constant agitation on an orbital shaker (50 rpm). IgG concentration was 5 μg/ml in 5 mL PBS-T. Following incubation with antibodies, microarray slides were washed 3 × 5 min with PBS-T with constant agitation (50 rpm). After, salts were removed by soaking the slides in 1/10 solution of PBS in deionized water and dried by centrifugation (100 × g) for 1 min. Fluorescence intensity at 532 nm and 635 nm was measured by the microarray scanner GenePix 4000B (Molecular Devices, San Jose, CA). Raw data from microarray chips were quantified and analyzed using Spotxel software v. 1.7.7 (Sicasys, Heidelberg, Germany).

Thermal shift assay for estimation of thermodynamic stability of IgG

To assess thermodynamic stability of human and bat (*M. myotis* and *M. capaccinii*) purified IgG molecules we used Protein Thermal Shift Dye Kit (Applied Biosystems, Thermo Fisher Scientific) following the manufacturer's protocol. All IgG samples were diluted in PBS and tested at a concentration of 80 μg/ml. Protein unfolding reactions and fluorescent signal detection were performed in Applied Biosystems 7900 HT Fast Real-Time PCR machine (Applied Biosystems, Thermo Fisher Scientific). For data analyses, we used GraphPad Prism v. 9.5 software (GraphPad Software, San Diego, CA). Melting points (T_m) of IgG molecules were calculated by fitting experimental data using the Boltzmann equation.

Fluorescence spectroscopy (ANS fluorescence)

To analyze the overall hydrophobicity of bat (*M. myotis*) and human IgG molecules, we used a well-established approach based on the fluorescent molecular probe 8-anilino-1-naphthalenesulfonic acid (ANS, Fluka, Sigma-Aldrich) which changes its fluorescence properties upon interaction with hydrophobic molecular environment in proteins. Purified polyclonal human and bat IgG was diluted to 0.5 μM (0.075 μg/ml) in PBS and titrated with ANS, resulting in final probe concentrations of 0, 0.5, 1, 2, 4, 8, and 16 μM. As a control, ANS was titrated in buffer only. Fluorescence measurements were performed by Hitachi F-2500 Fluorescence spectrophotometer (Hitachi, Tokyo, Japan). After adding each aliquot of ANS, reaction mixture was intensively homogenized for 1 min. After excitation with a wavelength of 388 nm, ANS emission spectra in the wavelength range of 400-600 nm were recorded. Fluorescence data were acquired at a rate of 300 nm/min. PMT voltage of the spectrophotometer was 400V and the excitation and emission slits were set to 10 nm. All measurements were performed in a quartz optical cell with an optical path of 10 mm at 22 °C.

Flow cytometry analyses

Human skin endothelial cell line HMEC-1 (ATTC, Manassas, VA) was cultured under standard conditions in a complete MCDB 131 cell culture medium (Gibco™, Thermo Fisher Scientific) containing 10 % fetal calf serum (ATCC), 10 ng/mL epidermal growth factor (EGF, Thermo Fisher Scientific), 1 pg/mL Hydrocortisone (Sigma-Aldrich), and 10 mM glutamine (ATCC). To create pro-apoptotic conditions, HMEC-1 cells were first starved in a serum free medium Opti-MEM™ (Gibco™, Thermo Fisher Scientific) for 24 hours, then they were exposed for 24 hours to Opti-MEM™ containing 1 mM of H_2O_2. To represent normal conditions, MCDB 131

medium was not changed. After the incubation, the cells were washed with PBS by centrifugation at 1600 rpm for 10 min at 4 °C. A sample of cells was stained with Trypan Blue (Gibco™, Thermo Fisher Scientific) to estimate the percentage of dead cells. Following washing with PBS, HMEC-1 cells were resuspended in PBS and distributed in FACS tubes with 10^5 cells per tube. For assessment of cell death by flow cytometry, a part of samples were labelled with annexin V-conjugated with APC and propidium iodide (Molecular Probes, Thermo Fisher Scientific). HMEC-1 cells cultured in pro-apoptotic and in normal conditions were distributed and incubated in the presence of Alexa Fluor™ 555-conjugated polyclonal human and bat (*M. myotis*) IgG at a concentration of 25 µg/ml. To assess the effect of temperature on IgG binding to live and dead cells, samples were incubated for 30 min at 22 or at 42 °C. Cells were then washed with PBS at 1,600 rpm, 4°C for 10 min and binding was analyzed by flow cytometer FACS LSRFortessa™ (BD Bioscience, Franklin Lakes, NJ) using Yellow/Green Ex 561 nm Em 586/15 laser for the detection of antibodies. Mean fluorescence intensity (MFI) indicating antibody binding was measured using FlowJo Software (LLC, Ashland, OR). The data was normalized by calculating the MFI ratio against the mean negative control.

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Figure Legends

Figure 1. Temperature modulates the antigen binding properties of bat IgG. **a.** Heat map depicting the binding intensity of IgG purified from pooled sera of *N. noctula, M. myotis* and healthy human (two different pools) to a panel of antigens. The reactivity of bat and human IgG was evaluated at 4, 22 and 42 °C by ELISA. The binding of IgG to the blocking agent (Bckg) is also shown. The binding intensity against each target represent average optical density (n=2) after subtraction of the background binding (measured after incubation of the detection reagents with the respective antigens in absence of antibodies). The violin plots corresponding to each heat map show the Z-scores values of antigen binding. The z-score for each target antigen is presented as an individual circle. *P* values were determined using one-way analysis of variance (ANOVA) with Friedman multiple comparison test, $*p < 0.05$, $***p < 0.001$, n.s., not significant. **b.** Real-time interaction profiles showing the binding as a function of temperature of IgG from *M. myotis* to immobilized on sensor chip Rabies virus glycoprotein and TBEV NS1 antigen. The antibodies were injected at serial dilution in concentration range 670 – 41.875 nM. The apparent binding affinities were evaluated by global kinetics analyses on results obtained at 42 °C. The global analyses kinetics fits are presented at **Suppl. Fig. 2**. **c**. Effect of temperature on antigen binding by bat and human IgG in whole sera. Pooled bat and human sera were serially diluted from 60 × to 1920 × and incubated with immobilized antigens at 4, 22 and 42 °C. Each data point represents average IgG binding intensity ±SD from n=3 technical replicates. **d**. Immunofluorescence analyses of binding of bat and human IgG to HEp-2 cells at 22 and 42 °C. The images are representative examples from n=9 fluorescence acquisitions from two independent experiments (magnification × 63). **e**.

Quantification of fluorescence intensity from immunofluorescence analyses of binding of bat and human IgG to Hep-2 cells. The graphs show the mean fluorescence intensity of n=9 images, for each condition, acquired in two independent experiments. Statistical analyses were performed by using nonparametric t-test, Mann-Whitney test, ****$p < 0.0001$.

Figure 2. Bat antibodies acquire broad reactivity towards diverse proteins at elevated temperature. The presented plots depict summarize results from Protein microarray analyses of binding of bat (*M. myotis*) and human pooled IgG antibodies to human proteins. In **a**. the antibody reactivity at 22 °C was plotted versus the reactivity at 42 °C; in **b**. the plots of reactivities of bat versus human IgG are shown. Each dot in a and b represent average MFI (n=2) signifying the binding of IgG to a single protein in the array, after substruction of the background reactivity. Red dashed lines represent the threshold of 1000 MFI, identifying the antibody specificities with high binding intensity. **c.** Plots depicting Z-scores of antibody binding reactivities to human proteins. Each dot in the plots represents the Z-score characterizing the binding reactivity to a single protein. The threshold indicated with red dotted lines corresponds to Z=2 and $p < 0.05$.

Figure 3. Bat IgG antibodies are characterized with untypical physiochemical characteristics. **a.** Graph depicting box plot with melting temperatures of bat (*M. myotis*) and human IgG, obtained by thermal-shift assay. Each dot in the plots indicate individual estimation of the melting temperature. The results of two independent experiments are presented together. The statistical significance was assessed by applying nonparametric t-test analyses, Mann-Whitney test. **$p = 0.0022$. Comparison of the thermodynamic stability of IgG from another bat species (*M. capaccinii*) with stability of human IgG is depicted in Suppl. Fig. 7. **b**. Changes of fluorescence intensity of 1-Anilinonaphthalene-8-Sulfonic Acid (ANS) obtained after titration of 0.5 μM of human and bat pooled IgG with increasing concentrations of the probe (0, 0.5-16 μM). Concentration-dependent change in the maximum fluorescence intensity for different samples is also depicted. Representative fluorescence spectra from three independent experiments are shown.

Figure 4. Bat antibody repertoires have higher level of polyreactive IgG antibodies. **a.** Heat map depicting the binding intensity of human polyreactive (ED38) and monoreactive (VRC01 and S309) IgG1 to a set of distinct antigens. The reactivity of monoclonal antibodies was evaluated at 4, 22 and 42 °C by ELISA. The binding of IgG to the blocking agent (Bckg) is also shown. The binding intensity against each target represent average optical density (n=2) after subtraction of the background binding measured after incubation of the detection reagents to the respective antigens in absence of IgG. For analyses all antibodies were diluted to 10 μg/ml. The violin plots corresponding to each heat map show the z-scores values of antigen binding. The z-score for each target antigen is presented as an individual circle. *P* values were determined using one-way analysis of variance (ANOVA) with Friedman multiple comparison test, $*p = 0.0153$, $**p = 0.0035$, n.s., not significant. **b.** and **c.** Binding of increasing concentrations of human and bat (*M. myotis* and *N. noctula*) IgG antibodies (b) and human monoclonal IgG1 antibodies (c) to immobilized DNP-BSA. Each data point on the graphs represents average optical density (n=3) ±SD. Representative results from two independent experiments are shown.

Figure 5. Temperature triggers preferential binding of bat antibodies to dead endothelial cells. **a**. Gating strategy and identification of induction of cell death of HMEC-1 cells by oxidative stress. The dead cells are defined as double positive for annexin V and propidium iodide. **b**. Binding of bat (*M. myotis*) and human pooled IgG to alive and dead HMEC-1 cells as a function of temperature. The binding of fluorochrome labeled (Alexa Fluor™ 555) pooled IgG at 25 μg/ml at 22 and 42 °C. Representative histograms from two independent experiments are shown. **c**. Quantification of the binding of bat and human IgG to alive and dead HMEC-1 cells. The ratio of MFI depicting the reactivity of antibodies was obtained from two independent experiment. Each data point in the graph represents MFI ratio from independent experimental replicate. Statistical significance was evaluated by applying non-parametric t-test, Mann-Whitney test. $*p = 0.0286$.

Figure 1

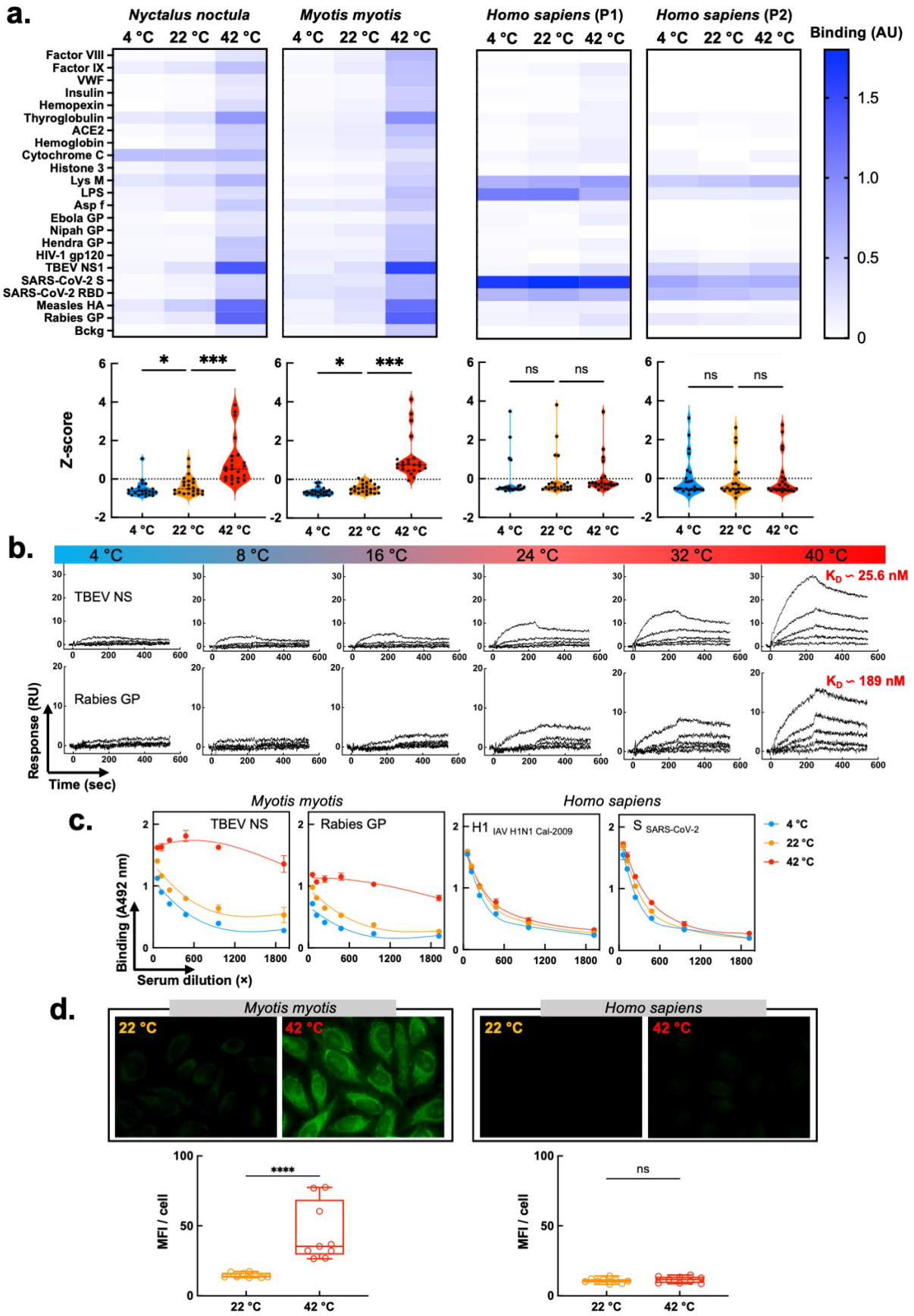

a.
Nyctalus noctula
Myotis myotis
Homo sapiens (P1)
Homo sapiens (P2)
4 °C 22 °C 42 °C
Binding (AU)
Factor VIII
Factor IX
VWF
Insulin
Hemopexin
Thyroglobulin
ACE2
Hemoglobin
Cytochrome C
Histone 3
Lys M
LPS
Asp f
Ebola GP
Nipah GP
Hendra GP
HIV-1 gp120
TBEV NS1
SARS-CoV-2 S
SARS-CoV-2 RBD
Measles HA
Rabies GP
Bckg
Z-score
ns
b.
4 °C 8 °C 16 °C 24 °C 32 °C 40 °C
TBEV NS
Rabies GP
K_D ~ 25.6 nM
K_D ~ 189 nM
Response (RU)
Time (sec)
c.
Myotis myotis
Homo sapiens
TBEV NS
Rabies GP
H1 IAV H1N1 Cal-2009
S SARS-CoV-2
Binding (A492 nm)
Serum dilution (×)
d.
Myotis myotis
Homo sapiens
22 °C
42 °C
MFI / cell
****
ns

Figure 2

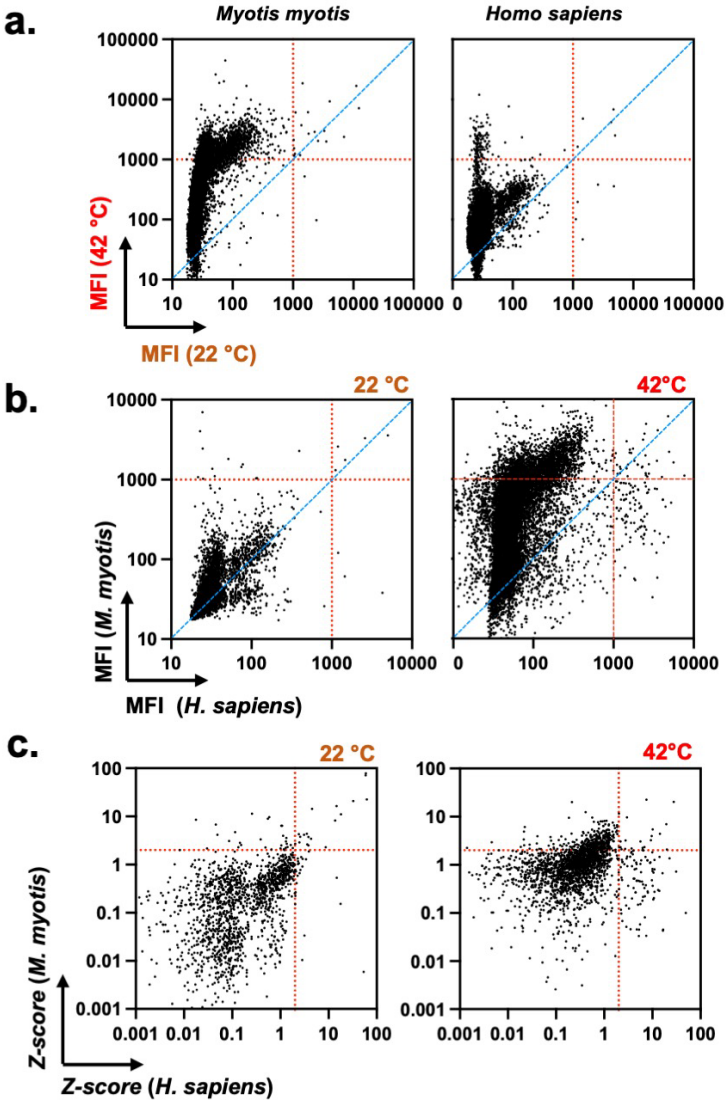

a.
Myotis myotis
Homo sapiens
MFI (42 °C)
MFI (22 °C)
b.
22 °C
42°C
MFI (M. myotis)
MFI (H. sapiens)
c.
22 °C
42°C
Z-score (M. myotis)
Z-score (H. sapiens)

Figure 3

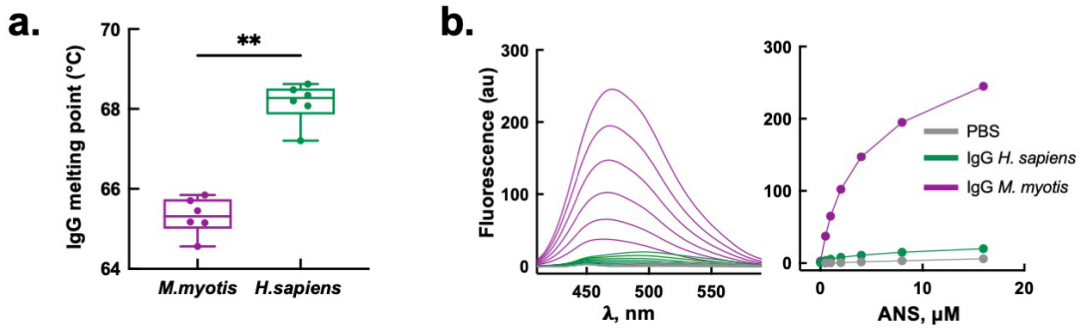

a.
IgG melting point (°C)
70
68
66
64
**
M.myotis
H.sapiens
b.
Fluorescence (au)
300
200
100
0
450
500
550
λ, nm
300
200
100
0
0
10
20
ANS, μM
PBS
IgG H. sapiens
IgG M. myotis

Figure 4

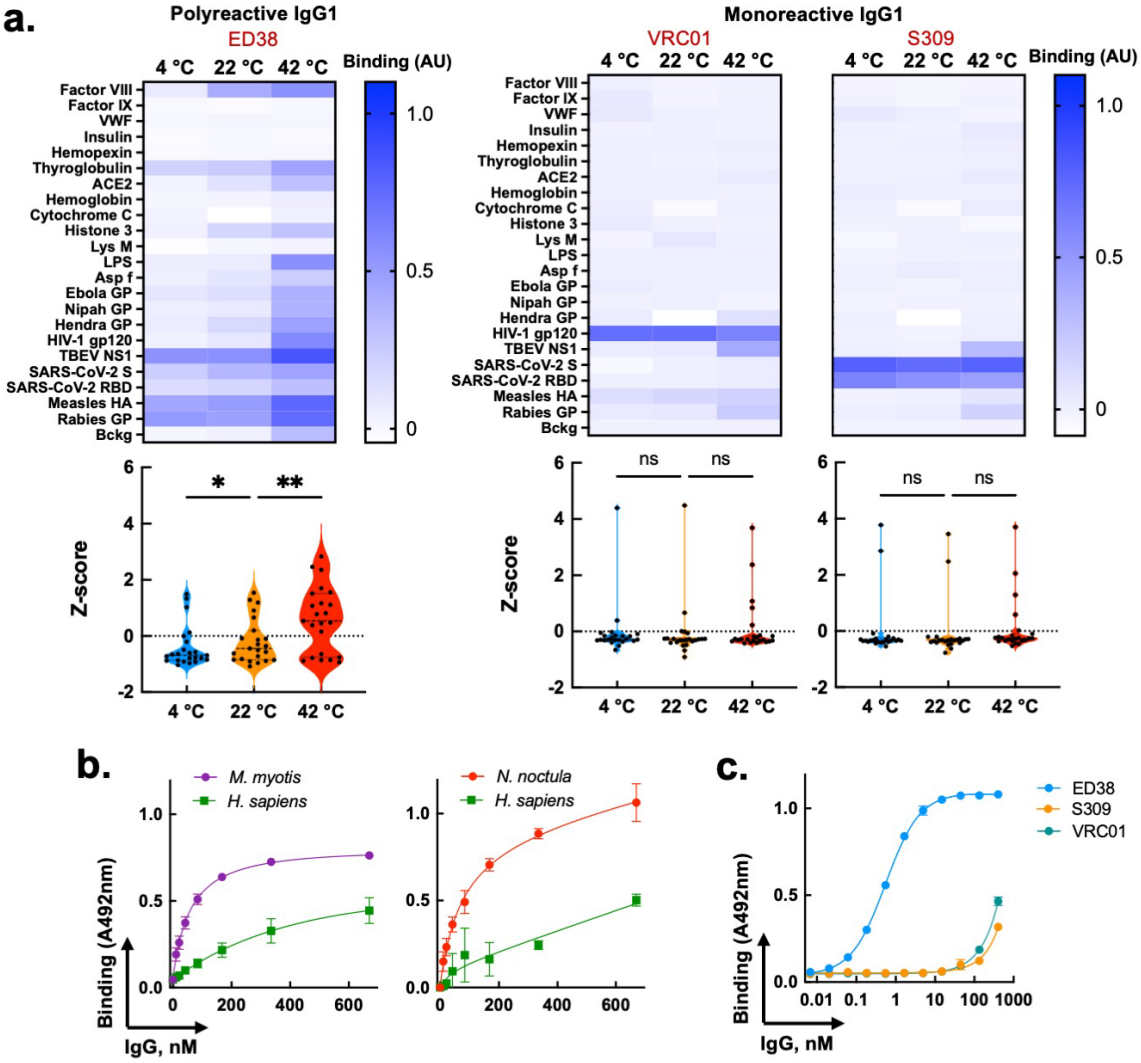
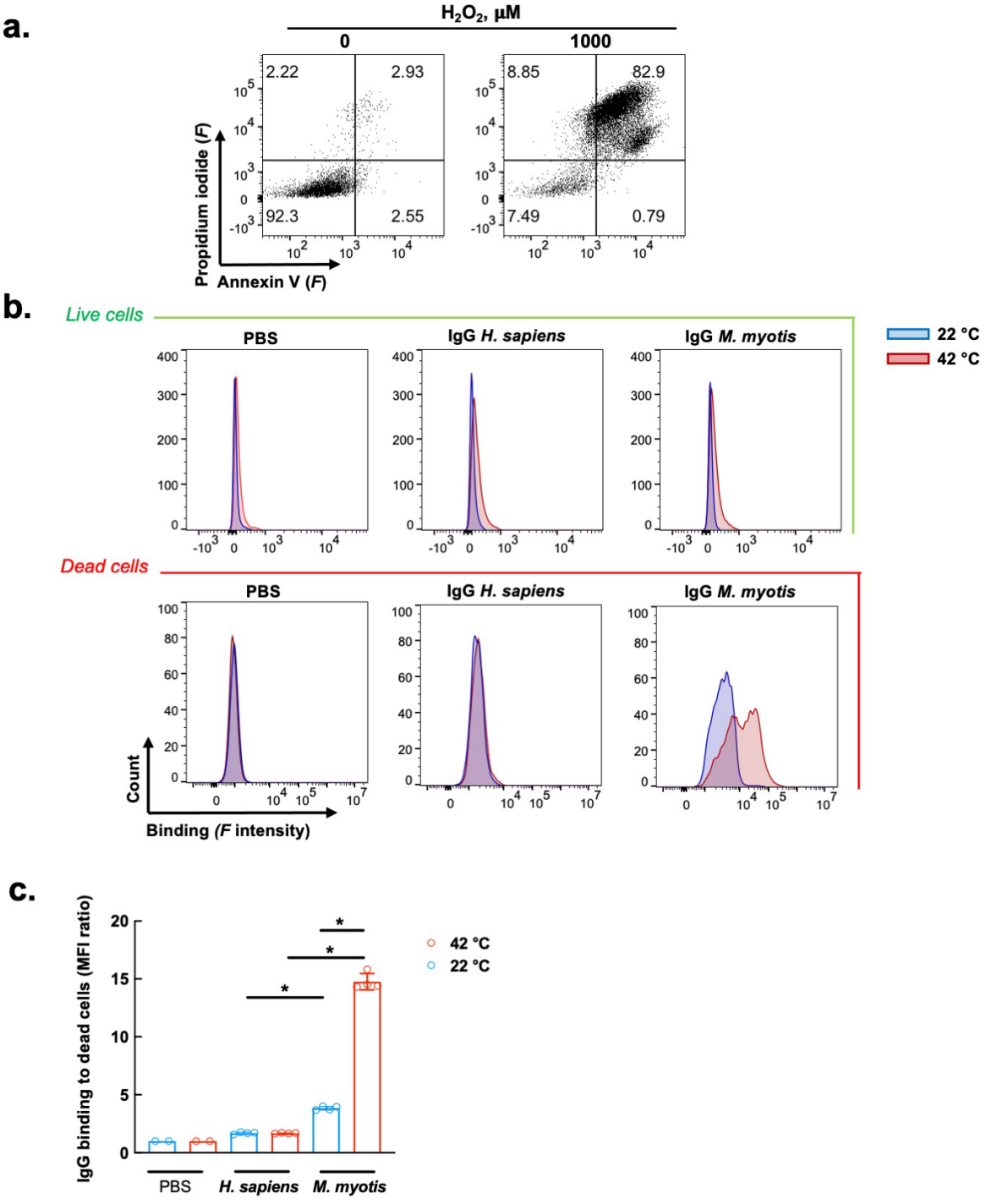

a.
Polyreactive IgG1
ED38
4 °C 22 °C 42 °C
Binding (AU)
Factor VIII
Factor IX
VWF
Insulin
Hemopexin
Thyroglobulin
ACE2
Hemoglobin
Cytochrome C
Histone 3
Lys M
LPS
Asp f
Ebola GP
Nipah GP
Hendra GP
HIV-1 gp120
TBEV NS1
SARS-CoV-2 S
SARS-CoV-2 RBD
Measles HA
Rabies GP
Bckg
1.0
0.5
0
Monoreactive IgG1
VRC01
S309
Z-score
*
**
ns
b.
M. myotis
H. sapiens
N. noctula
Binding (A492nm)
IgG, nM
c.
ED38
S309
VRC01

Figure 5


a.
H2O2, µM
0
1000
2.22
2.93
92.3
2.55
8.85
82.9
7.49
0.79
Propidium iodide (F)
Annexin V (F)
b.
Live cells
Dead cells
PBS
IgG H. sapiens
IgG M. myotis
22 °C
42 °C
Count
Binding (F intensity)
c.
IgG binding to dead cells (MFI ratio)
42 °C
22 °C
PBS
H. sapiens
M. myotis