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Research paper

High incidence of anti-cytokine autoantibodies in dogs with immune diseases suggests important immuno-regulatory functions

M. Autran-Martínez[a], P. Cortes-Acevedo[a], L. Cerón-Gutiérrez[b], S. Davies[b], E.S. Mendoza-Elvira[f], H.A. Martínez-Rodríguez[c], C. Liboni[d], V. Pérez-Valencia[e], R. Döffinger[b,*], G. Barcenas-Morales[a]

[a] Laboratorio 2 Inmunología, Facultad de Estudios Superiores (FES) Cuautitlán, Universidad Nacional Autonoma de México (UNAM), Mexico
[b] Department of Clinical Biochemistry and Immunology, Addenbrookes Hospital and National Institute of Health Research (NIHR), Cambridge Biomedical Research Centre, Cambridge, UK
[c] Laboratorio de Virología, Genética y Biología Molecular, FES Cuautitlán, UNAM, Mexico
[d] Universitá degli studi di Padova, Dipartimento di Scienze Biomediche- Via Ugo Bassi, 58/B-35131 Padova Italy Fondazione Istituto di Ricerca Pediatrica Cittá della Speranza-Corso Stati Uniti, 4-37127, Padova, Italy
[e] Hospital Veterinario Belén, Tultitlán, Estado de México, Mexico
[f] Laboratorio de Microbiología. FES Cuautitlan, UNAM, Estado de México, Mexico

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ABSTRACT

Autoantibodies against cytokines have been associated with immunodeficiency, susceptibility to infectious diseases, autoimmunity and inflammation in humans, but have not yet been investigated in the Veterinary field so far. The aim of the current study was to determine the presence of anti-cytokine autoantibodies in canines suffering from various conditions including recurrent infections, autoimmune diseases and cancer in comparison to healthy controls. This is the first report of the presence of autoantibodies against cytokines in dogs. A total of 101 serum samples (51 patients and 50 clinically healthy dogs) from the state of Mexico and surroundings were analysed using a multiplex bead-based flow cytometry assay. Results show significant levels of various anti-cytokine autoantibodies in diseased dogs but not in healthy controls. In addition we show distinct associations of various disease types to the specificity of anti-cytokine autoantibodies and to response complexities. Apart from the direct functional/causal implication of anti-cytokine auto-antibodies on disease processes, this findings point to the possibility to use anti-cytokine response patterns as diagnostic tools.

1. Introduction

The immune system defends organisms against pathogens. Antibodies are major effectors of the adaptive immune response. Higher organisms such as vertebrates have developed mechanisms of tolerance in order to focus its immune responses to 'foreign' pathogen-derived antigens and to avoid responses against 'self' antigens. Sometimes, the state of self-tolerance may be broken resulting in autoimmune disease (Schwartz, 2012). However autoantibodies may as well have regulatory functions (Silverman et al., 2013). Cytokines are secreted proteins, which are key mediators of immune responses. They play an important role in the organization of the host defense against pathogens. Cytokine deficiency may cause susceptibility to infection and/or immune dysregulation. Overproduction of proinflammatory cytokines may cause various inflammatory diseases (Dinarello, 2007). A steadily increasing number of studies in humans identified high titer often functionally neutralizing autoantibodies to single cytokines alone or as part of more complex anti-cytokine patterns to be associated with immunodeficiency characterized by susceptibilities to various pathogens (Browne, 2014; Barcenas-Morales et al., 2016; Knight et al., 2016; Barcenas-Morales et al., 2019). This was recognized as secondary autoimmune phenocopies of primary immune deficiencies (Picard et al., 2018). Autoantibodies have been proposed to be as well involved in preventing infection, maintenance of physiological homeostasis and immune

Abbreviations: α, prefix of anti (against); ACAB, anti-cytokine autoantibodies; AID, autoimmune disease; APECED, autoimmune-poly-endocrinopathy-candidiasis-ectodermal-dystrophy syndrome; ID, infectious disease; Ca, cancer; EBA, epidermolysis bullosa acquisita; GF, growth factors; GM-CSF, Granulocyte/Macrophage Colony Stimulating Factor; G-CSF, Granulocyte Colony Stimulating Factor; IBD, inflammatory bowel disease; IMUK, immune-mediated ulcerative keratitis; MG, myasthenia gravis; MFI, mean fluorescence intensity; PAP, pulmonary alveolar proteinosis; PCA, principal component analysis; RA, rheumatoid arthritis; rHu, recombinant human; SLE, systemic lupus erythematous

* Corresponding author.
E-mail address: rd270@medschl.cam.ac.uk (R. Döffinger).

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regulation (Wildbaum et al., 2003; Watanabe et al., 2007; Lutz et al., 2009; Browne, 2014; Barcenas-Morales et al., 2016; Knight et al., 2016; Barcenas-Morales et al., 2019). Anti-cytokine autoantibodies have so far been mostly studied in humans. Here, we investigated the presence of serum ACAB in domestic dogs to test the hypothesis if there is an association with various pathological conditions including recurrent infections, autoimmune diseases, and cancer. The study focused on evaluating canines selected from private veterinary clinics, shelters and the veterinary hospital at the FESC, UNAM that were grouped according to disease manifestations.

2. Material and methods

2.1. Study population

Serum samples from diseased dogs (> 1 year up to 9 years, n = 51) and healthy control dogs (> 1 year up to 8 years, n = 50) were obtained after informed owner consent. The study was approved by the Internal Committee for Care of Experimental Animals of the Postgraduate Program of Animal Production and Health (UNAM, Mexico) and was carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, of the National Institutes of Health guide. Control subjects were considered to be clinically healthy based on history, physical examination, complete blood count, and biochemistry panel. Medical records were obtained from private veterinary clinics, shelters and the veterinary hospital at the FES, UNAM. Data regarding age, gender, daily outdoor access, origin, vaccination history, clinical biochemical profiles, serological tests and complete hematological panels were recorded.

2.2. Disease categories

The patient cohort were divided into three main groups according to their disorders: **1)** Autoimmune diseases including systemic lupus erythematous (SLE), pemphigus, immune-mediated ulcerative keratitis, rheumatoid arthritis, myasthenia gravis, epidermolysis bullosa acquisita, thyroiditis, inflammatory bowel disease (IBD) and myositis; **2)** Infectious diseases including recurrent and/or severe infections caused by canine distemper virus, intracellular bacteria (*Salmonella, Escherichia coli, Bordetella bronchiseptica*), fungi (*Histoplasma capsulatum,* various Dermatophytes*),* generalized dermatitis by *Staphylococcus intermedius and Streptococcus pyogenes and secondary Staphylococcus aureus*, recurrent otitis with secondary *Malassezia pachydermatis*, gastroenteritis by enterobacteria such as P*roteus* and *E. Coli;* and **3)** Cancer including lymphoma or acute myeloid leukemia.

2.3. In silico sequence comparison of human and canine cytokines

Considering that the commercial availability of canine cytokines and related reagents is limited (Levin et al., 2014), we decided to use human cytokines. It was necessary to demonstrate that canine cytokines are sufficiently homologous (percentage of similarity and divergence) to their human counterparts in order to allow a substantive and informative polyclonal cross recognition. Homology analysis and identity matrix were performed using the GenBank database NCBI® and Protein Data Bank PDB®, the Bioedit® (Hall, 2001) and MEGA 6® (Tamura et al., 2013), allowing to analyze, recognize, and compare the cytokine peptide sequences of these two different species.

2.4. Coupling of anti-cytokine beads for multiplexed bead array

Recombinant human cytokines (IFN-α, IFN-β, IFN-ω, IFN-γ, IFN-λ, IFN-κ, IL-17A, IL-17 F, IL-21, IL-22, IL-23, IL-2, IL-6, IL-10, IL-1α, IL-28A, IL-28B, IL-15, IL-27, TGFβ, TNFα, IL-12, IL-1α, IL-1β, IL-8, TNFα, Granulocyte/Macrophage Colony Stimulating Factor (GM-CSF), Granulocyte Colony Stimulating Factor (G-CSF) and Bovine serum albumin (BSA control), (R&D Systems, Minneapolis, Minn., Table 1) were covalently coupled to carboxylated beads (Bio-Plex; Bio-Rad Laboratories, Hercules, California) as previously described (Rosenberg et al., 2016). Briefly, for the coupling procedure, the bead was activated with 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC; Thermo Fisher Scientific, Waltham, Mass Cat. Nu. E6383) in the presence of *N*-hydroxysuccinimide (NHS; Thermo Fisher Scientific Cat. Nu. 606682-6), according to the manufacturer's instructions to form amine-reactive intermediates. The activated bead was then incubated with the recombinant human (rHu) cytokine (20 µg/mL) for 3 h at 37 °C on a rotator in darkness. The coupled bead was washed, counted to a concentration of 2×10^6 beads/mL and stored in blocking buffer (FBS 1%, Sigma-Aldrich Cat. Nu. 997RA) in Phosphate Buffered Saline (PBS; Gibco, Cat. Nu. 10010023) and 0.05% Sodium Azide NaN_3 (Sigma-Aldrich, Cat. Nu.26628-22-8).

Table 1
Homology between human and canine cytokines.

Human/ Canine cytokine	Homology (%)	GenBank Access Protein Number Human cytokine	GenBank Access Protein Number Canine cytokine
IL-17A	99.1	NP_002181.1	UPI0001B05147
G-CSF	99.0	AJC19277.1	1BGD_A
IL-6	98.9	P05231.1	NP_001003301.1
IFN-γ	98.6	NP_000610.2	NP_001003174.1
IL-10	98.3	P48411.1	ABY86619.1
IL-17F	97.6	AAC50341.1	BAH89243.1
IL-23	97.2	NP_057668.1	NP_112542.1
IL-21	97.0	NP_001193935.1	NP_001003347.1
IL-22	96.9	AAH69308.1	XP_025332317.1
GM-CSF	95.6	AAA52578.1	NP_001003245.1
IFN-λ	92.8	NP_742152.1	AGN92945.1
IFNβ	92.4	NP_002167.1	AYN61080.1
IL-2	88.0	AAB46883.1	NP_001003305.1
IL-28	79.0	AAR24510.1	NP_742152.1
IL-27	77.6	AAH62422.1	NP_663611.1
IL-15	76.0	NP_001184117.1	NP_001184117.1
IL-12	64	AAB36675.1	NP_001003293.2
IL-8	41.6	NP_001341769.1	NP_001003200.1
IFN-α	19.3	AAA52724.1	AAA30852.1
IFN-ω	9.7	CAA26501.1	AAB27160.1.3

The successful coupling of the rHu-cytokine to its respective bead set was verified with specific mAb (R&D Systems, Minneapolis, Minnesota). BSA-control beads where run for patient and control sera and found to be negative (data not shown).

2.5. Detection of anti-cytokine autoantibodies in canine sera using multiplexed microparticle-based flow cytometry

Blood Samples were obtained by puncture of the jugular or radial veins from patients and normal donors, and sera were obtained using tubes without anticoagulant (Vacutainer BD® Mexico). Following coagulation and subsequent centrifugation (10 min, 2500 X *g*) samples were stored at −20 °C. For the assay the 30 coupled cytokine-beads sets were mixed and incubated with diluted sera (1/200) from patients and healthy controls for 30 min in a 96-well filter plate (Multi Screen HTS; Millipore, Temecula, California Cat. Nu. PF1150EN00) at 37 °C in the dark on a horizontal shaker. Fluids were aspirated with a vacuum manifold, and beads were washed 3 times with PBS/0.05% Tween 20 (Sigma, P2287). Following an incubation for 30-min with a phycoerythrin-labelled anti-dog IgG-Fc antibody 1/200 (Goat anti-canine IgG-PE, sc-3731, Cat. Nu. A18765, Santa Cruz Biotechnology®), beads were washed as described above and resuspended in 130 µL of PBS/ Tween. Samples were then analyzed on a Bio-Plex platform by using Bio-Plex Manager 6.1 software (Bio-Rad Laboratories).

2.6. Statistical analysis

The data was analyzed using the GraphPad® Prism software version 7.0 for Windows. The data set values where analyzed by means of ANDEVA, Mann-Whitney U test as unpaired non- parametric test. In order to visualize the fusion distances between the groups of patients in comparison with the clinically healthy subjects, a cluster dendrogram was performed with the program R commander i386 version 3.5.1 using the Ward method and a Euclidean distance (Fox and Bouchet-Valat, 2019). Based on the values obtained from healthy subjects (MFI ± 4 SD), a fluorescence intensity cut-off value (FI) of < 7000 was established and positive samples were therefore defined as ≥7000. A principal component analysis (PCA) was carried out using XLSTAT software (Addinsoft, 2010), to be able to explore the relationship between the spectra of anti-cytokine autoantibodies and the different types of diseases. Correlations were calculated using the Pearson coefficient correlation and "*r*" value were interpreted as strong correlation to very weak correlation. An unsupervised hierarchical grouping was performed by means of the Pearson correlation with an average link grouping with the Multiple Experiment Viewer program MeV 4_9_0® for Windows 7 (Saeed et al., 2003), the level of statistical significance was established at $p \leq 0.005$.

3. Results

3.1. Homology between human and canine cytokines

An *in silico* analysis confirmed very high homology between most (but not all) canine cytokines and their respective human counterparts suggesting that human cytokines will allow to analyze canine anti-cytokine responses. We choose 16 target cytokines with at least 75% homology (mean homology 93%), and as well 4 targets with lower homology, IL-12 (64%), IL-8 (41%), IFNα (19.3%) and IFNω (9.7%) as the latter had been previously reported to be targets of ACAB (Rosenberg et al., 2016). We used BSA-coupled control beads for background evaluation, which showed negative results for controls and patients (data not shown). Most of the selected targets however exhibited homologies of above 90% (14 out of 20, mean homology 96%) (Table 1).

3.2. Detection of anti-cytokine autoantibodies in dog sera using multiplexed microparticle-based flow cytometry

In the current study, we wanted to investigate if anti-cytokine autoantibodies are present in the sera of dogs. We collected serum samples from dogs presenting with three main groups of diseases (autoimmune disease, infectious disease, and cancer) referred from throughout Mexico (n = 51) and compared them to clinically healthy subjects (n = 50) from different breeds without known congenital disease (Table 2). Anti-cytokine autoantibodies to 30 cytokines were measured by multiplexed bead array on a Luminex system. The anti-cytokine data from the 50 healthy control subjects were then used to define cytokine specific cut-offs. Individual mean fluorescence intensities (MFI) values were found to be between 83 and 1650; the cut-off values were calculated based on MFIs ± 4 SD, which were set in a range of 827 to 9938. A generalized arbitrary cut-off value of < 7000 was defined, which represents the mean of all cytokine MFI's ± 4 SD. Based on this cut-off value, 46 patients were found to be positive for IgG autoantibodies against at least one cytokine and 43 samples showed high MFI values (≥15,000) indicating elevated levels of IgG autoantibodies to 12 different cytokines (IFN-γ, IFN-λ, IL-17A, IL-17 F, IL-21, IL-22, IL-23, IL-2, IL-6, IL-10, GM-CSF and G-CSF). Results are shown as heat maps (Fig. 1, **Panel A**: Healthy controls and **Panel B**: Patients) and reveal individual patient-specific anti-cytokine reactivities. Some cytokines were targeted with higher frequencies, including in particular IFNγ (n = 19), IL-10 (n = 16), IL-17A (n = 15), IL-17 F (n = 11), IL-22(n = 4) followed by IL-6 (n = 5) and IFNλ (n = 5) (Fig. 1, **Panel B**).

Table 2
Clinical characteristics of the cohort of dogs.

Disease	Number	Gender		Status		
		Male	Female	Castrated	Spayed	Unknown
1) Autoimmune diseases						
Systemic Lupus Erythematous	5	1	4	1	2	2
Pemphigus	4	3	1	1	1	2
Myositis	3	1	2	0	1	1
Immune-mediated ulcerative keratitis	5	3	2	2	1	2
Rheumatoid Arthritis	2	1	1	1	0	1
Thyroiditis	2	0	2	0	1	1
Myastenia Gravis	2	0	2	0	1	1
Epidermolysis bullosa acquisita	3	3	0	2	0	0
Inflammatory bowel disease	4	3	1	1	1	0
Myositis	2	1	1	1	0	0
2) Infectious diseases						
Extracellular bacterial infection	1	0	1	0	0	0
Intracellular bacterial disease	3	0	2	0	1	0
Fungal infection	9	3	7	2	4	2
Viral infection	4	3	1	1	0	0
3) Other disease						
Cancer	2	1	1	1	1	0
Total Number of patients:	**51**	23	28	13	14	12
4) Healthy subjects	**50**	23	27	12	10	5

3.3. Significant anti-cytokine autoantibody responses in patients

An ANOVA analysis was performed comparing healthy controls and patients to evaluate differences in their ACAB's. Targets were divided into three different groups: Interferons, TH17 cytokines and other cytokines, corresponding to Fig. 2a, b and c, respectively. Significantly positive anti-cytokine responses were identified in the patients when compared to the control group, with the lowest *P* values identified for IFNγ (AID, $p < 0.00003$), IFNλ (AID, $p < 0.0001$), IL-17A (AID p 0.000002; ID, $p < 0.0001$), IL-17F (AID $p < 0.0001$), IL-6 (AID, $p < 0.0008$), IL-10 (AID, $p < 0.0001$; ID, $p < 0.00001$)

In addition, we performed unsupervised hierarchical clustering on the 15 highest targeted cytokines (Fig. 3, **Panel A**). Data reduction using a two-class significance analysis was performed and seven statistically significant differences were identified within the ACAB reactivity's when compared to the healthy controls: αIFNγ ($p = 0.0001$), αIFNλ ($p = 0.002$), αIL-17A ($p = 0.00003$), αIL-17 F ($p = 0.001$), αIL-22 ($p = 0.0003$), αIL-6 ($p = 0.002$) and αIL-10 ($p = 0.00004$) (Fig. 3, **Panel B**). A PCA was done to further analyze the anti-cytokine profiles among all the data set. Serum samples from patients and healthy subjects exhibited remarkably different responses against the targeted cytokines (Supplementary Fig. 4). A correlation matrix identified a strong correlation value ($r = 0.864$) between the patient´s disease and the targeted cytokine.

3.4. Disease specific anti-cytokine autoantibody reactivities

AID patients displayed more complex anti-cytokine responses with 27% showing high positivity to more than three cytokines versus only 6.25% in the ID group. Patient with skin related AID disease (5 out of 7) showed a particular tendency for αTH17 autoantibodies including

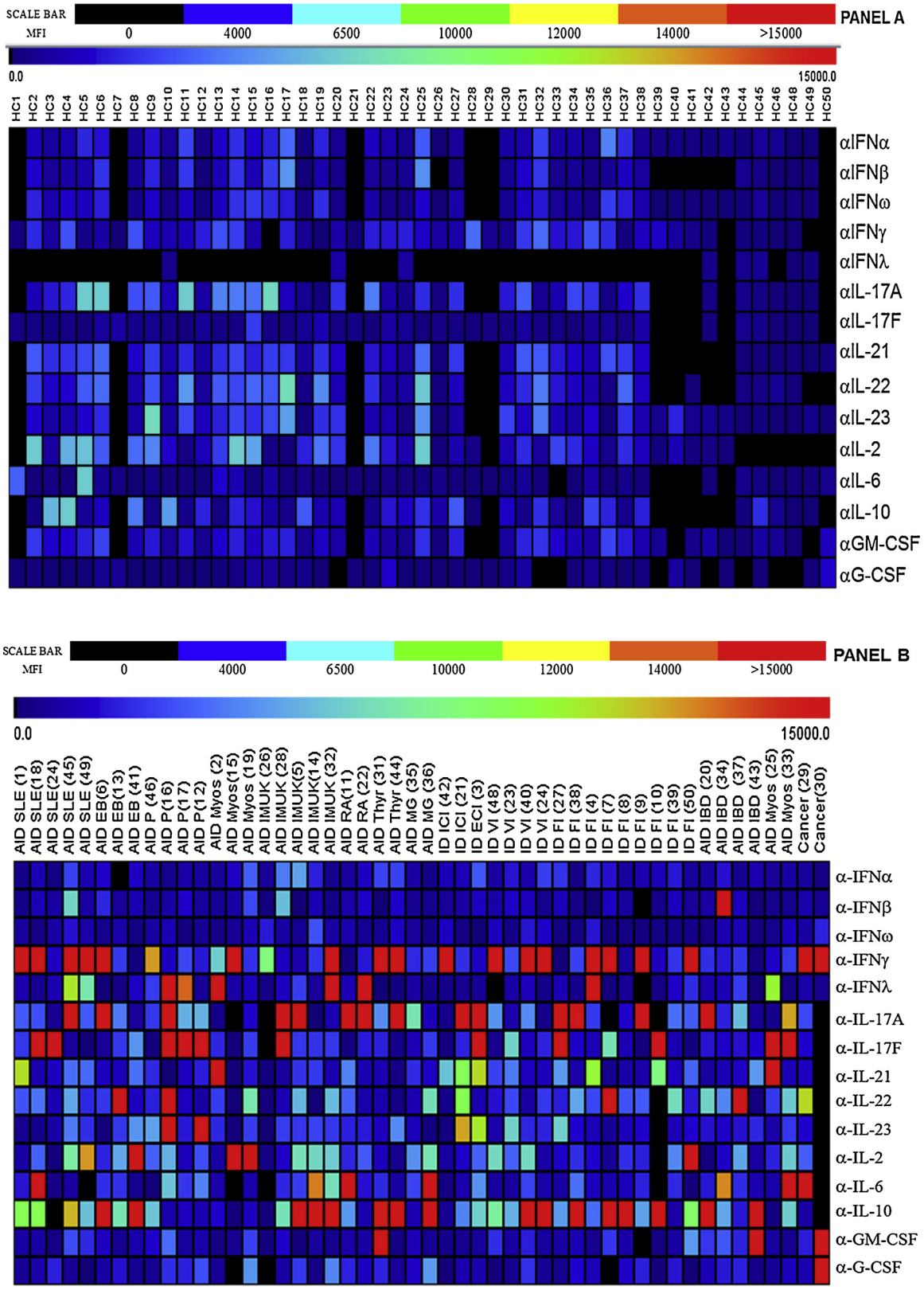

Fig. 1. Heatmaps of individual anti-cytokine values. Samples were plotted along the X-axis and the anti-cytokine autoantibodies were plotted along the Y-axis. MFI values from 0 to greater than 15000 are shown in the heatmap as a color scale ranging from black to red respectively. Anti-cytokine reactivity from the 50 healthy control (**Panel A**) subjects and 51 patients (**Panel B**) are shown. (α): Prefix of anti / against; (MFI): Mean fluorescence intensity.

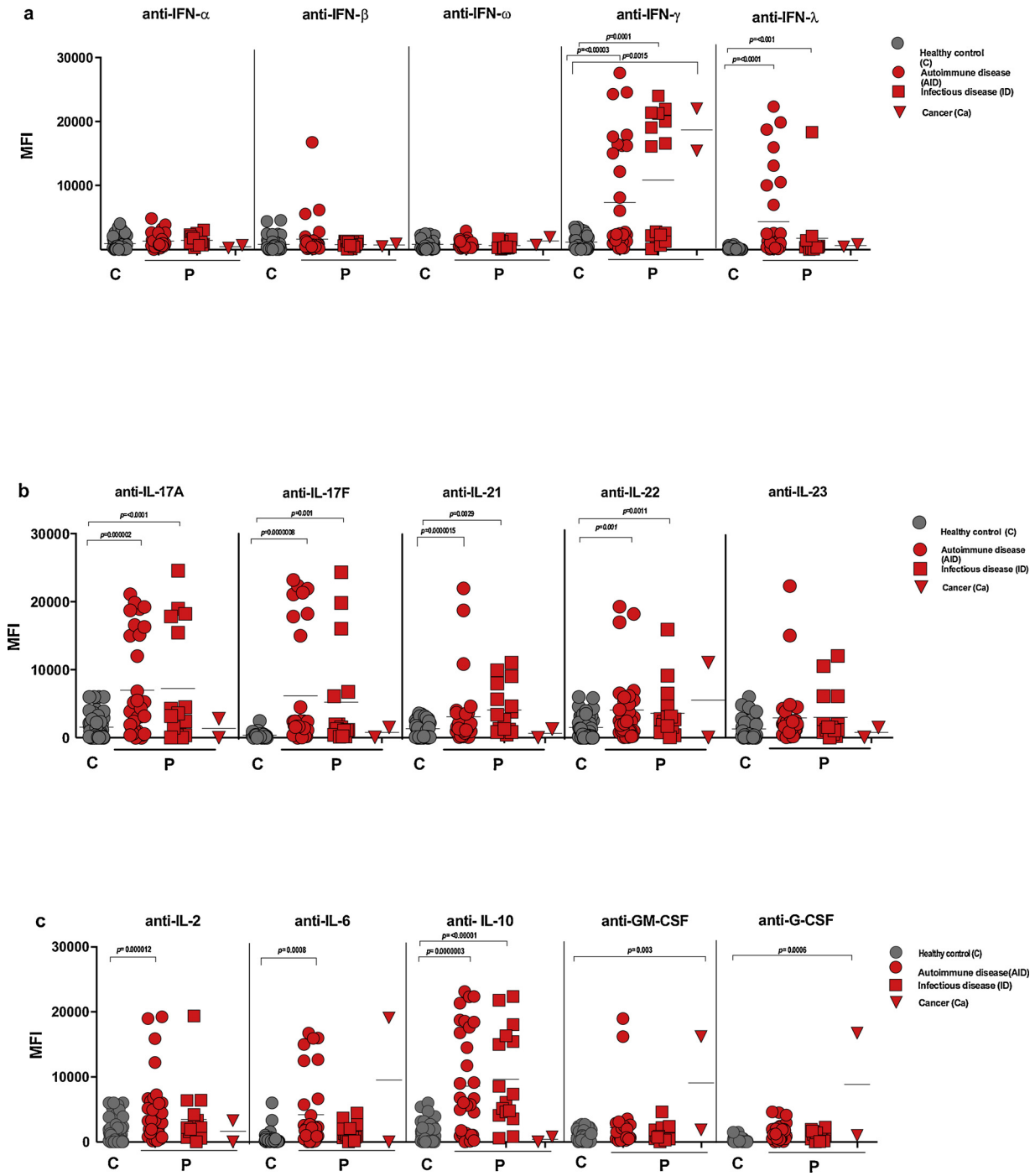

Fig. 2. a, b, c: Anti-cytokine autoantibodies in patients and healthy controls. Median fluorescence intensities (MFI´s) are shown: Healthy subjects (C, n = 50), Patients with autoimmune disease (P-AID, n = 32), Patients with Infectious disease (P-ID, n = 17), Patients with cancer (P-Ca, n = 2); Mean values are indicated as horizontal bars. P-values were calculated using two-tailed unpaired *t*-test Fig. 2a: autoantibodies to interferons - type I (IFNa, IFNb, IFNw) Type II (IFNg) and Type III (IFNl): Fig. 2b: autoantibodies to TH17 cytokines (IL-17A, IL-17 F, IL-21, IL-22, IL-23). Fig. 2c: autoantibodies to cytokines (IL-2, IL-6, IL-10, GM-CSF, G-CSF).

IL17A, IL17F and IL-22. Overall AID patient had a higher tendency to present with anti-Th17 antibodies when compared to ID patients (61% *vs.* 43% respectively).

Interestingly, two of the four patients with IBD displayed autoantibodies to IL-10. Other targets included the Th17 cytokines IL-17A and IL-22. One IBD patient showed autoantibodies to GM-CSF and one had antibodies to IFNβ, which was the only time, we found this activity in our population. From the 5 patients with autoimmune keratitis, 3 showed high activity against IL-10 and 2 against IL17-A and F. Major targets in SLE patients were IFNγ (4 out of 5) and IL-17A/F (3 out of 5) with one patient being positive for anti-IL-6. All dogs with thyroiditis displayed autoantibodies to IFNγ. The predominant targets in ID patients (n = 16) were IFNγ (8), IL-10 (6), IL-17A (5), IL-17F (3). Further targets include IL-22 and IFN-λ. Interestingly, some patients exhibited rare reactivity's: Both patients with myositis had antibodies to IL-21. Both cancer patients displayed relative unique patterns with antibodies to IL-6 and IFNγ, in one case with B-cell leukemia and G-CSF, GM-CSF and IFNγ in the second case with advanced multicentric B-cell lymphoma.

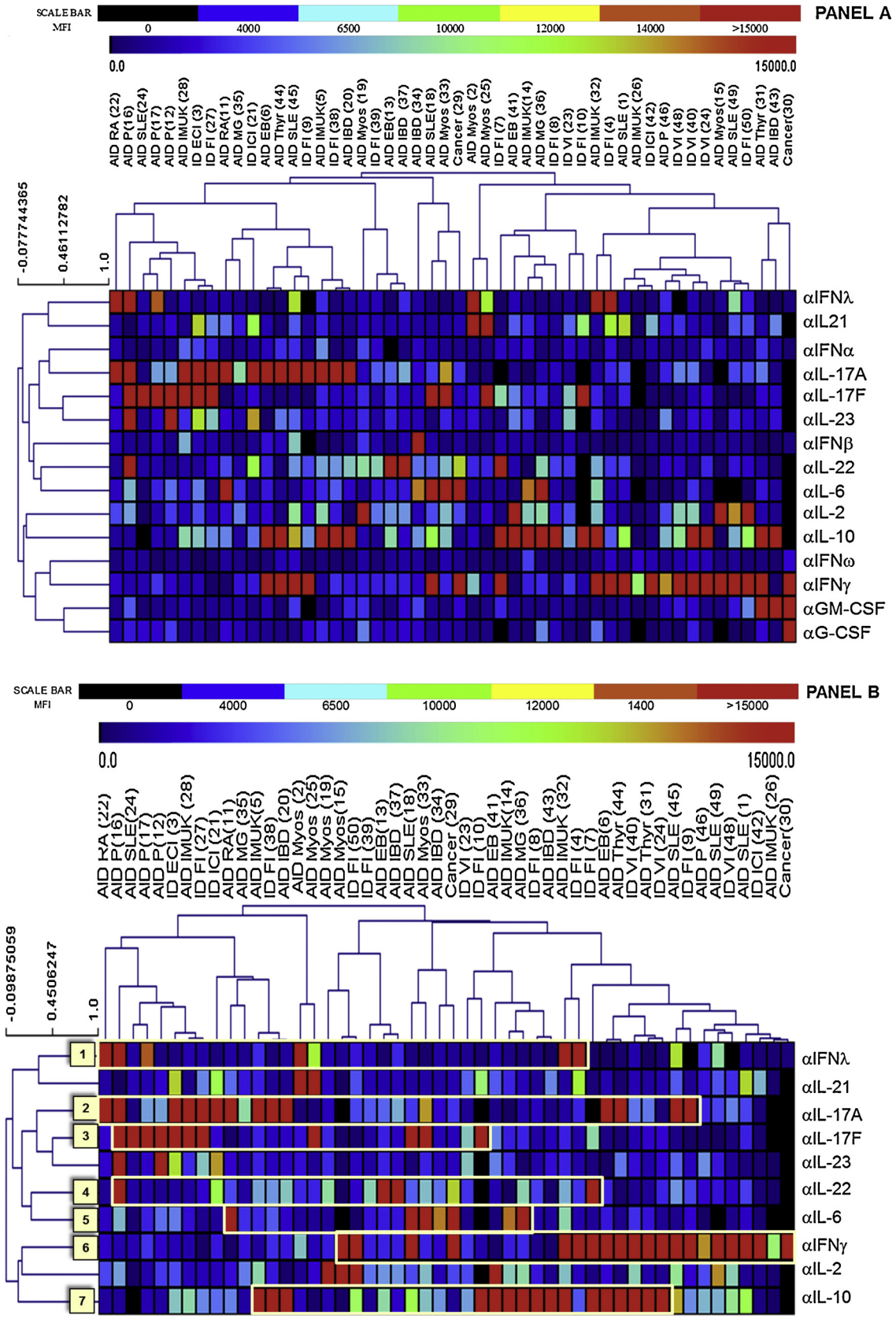

Fig. 3. PANEL A and B. Heatmaps of individual anti-cytokine values in patients. A dendrogram clustering using Euclidean distance as the metric, with related patterns shown in closer proximity. **Panel A:** Hierarchical clustering indicating reactivity patterns against 15 different cytokines in patients with several types of disease (n = 51) as probed by Luminex assay. Samples were plotted along the X-axis and the anti-cytokine autoantibodies were plotted along the Y-axis. FI values from 0 to greater than 15000 are shown in the heatmap as a colour scale ranging from black to red respectively. **Panel B.** A two-class significance analysis identifies seven statistically significant differences in a groups of 10 selected anti-cytokine reactivity's compared to healthy controls: different patterns of high anti-cytokine reactivity are shown: 1) anti-IFNλ autoantibodies ; 2 and 3) anti-IL17 A/ F ; 4) anti-IL-22 autoantibodies; 5) anti-IL-6 autoantibodies; 6) anti-IFNγ autoantibodies,7) anti-IL-10 autoantibodies. (α): Prefix of anti / against; (MFI): Mean fluorescence intensity.

4. Discussion

Autoantibodies to cytokines have been mainly studied in humans so far. They are increasingly recognized to cause acquired immunodeficiency and susceptibility to infection (Browne, 2014; Barcenas-Morales et al., 2016; Barcenas-Morales et al., 2019). In inflammatory conditions, they may be either protective or disease exacerbating (Knight et al., 2016; Barcenas-Morales et al., 2019). Anti-cytokine autoantibodies are also commonly found in healthy individuals and may play a role in the normal cytokine homeostasis (Watanabe et al., 2007; Lutz et al., 2009; Watanabe et al., 2010). Here we present the first study of anti-cytokine autoantibodies in the veterinary field. Using multiplexed bead arrays, we investigated the presence of serum ACAB to 21 cytokines in 101 domestic dogs (51 patients and 50 healthy controls) to test the hypothesis if there is an association with various pathological conditions including recurrent infections, autoimmune diseases and cancer. Dog anti-cytokine antibodies are mostly unavailable. We therefore used human cytokines as targets as most (but not all) dog cytokines showed very high sequence homologies to their human counterparts (Table 1).

4.1. Significant serum anti-cytokine autoantibodies in diseased dogs

We detected significant levels of anti-cytokine autoantibodies in patient dogs when compared to healthy control dogs. 46 patients were found to be positive for IgG autoantibodies against at least one cytokine and 43 samples showed high MFI values ($\geq$ 15,000) indicating elevated levels of IgG autoantibodies to 12 different cytokines including IFN-γ, IFN-λ, IL-17A, IL-17 F, IL-21, IL-22, IL-23, IL-2, IL-6, IL-10, GM-CSF and G-CSF (Fig. 1, **Panel A**: Healthy controls and **Panel B**: Patients) with patient-specific anti-cytokine reactivity patterns. Some cytokines were targeted with higher frequencies, including in particular IFNγ (n = 19), IL-10 (n = 16), IL-17A (n = 15), IL-17 F (n = 11), IL-22(n = 4) followed by IL-6 (n = 5) and IFNλ (n = 5) (Fig. 1, **Panel B**).

Two different statistical analytical approaches were applied. Using an ANOVA approach, significant positive anti-cytokine responses were identified in the patients when compared to the control group, with the lowest *P* values identified for IFNγ, IFNλ, IL-17A, IL-6, and IL-10 (Fig. 2a, b, c). Unsupervised hierarchical clustering revealed seven statistically significant differences within the ACAB reactivity's when compared to the healthy controls: including αIFNγ, αIFNλ, αIL-17A, αIL-17 F, αIL-22, αIL-6 and αIL-10 (Fig. 3, **Panel B**).

Some of the main targets identified in this study overlap with previously identified targets in human diseases. IL-17A/F, IL-22 and IFNλ have been previously identified as 'common' targets in patients with APECED and Thymoma and antibodies to IL-17A/F and IL-22 have in particular been associated with susceptibility to candidiasis (Kisand et al., 2010; Puel et al., 2010). We identified anti TH17 antibodies in 7 out of 16 dogs with ID, presenting with fungal (5) and bacterial infections (2). Candidiasis is however not a frequent manifestation in immunocompetent dogs (Krohne, 2000). We did not find autoantibodies to the type I IFNs IFNα and IFNω in our study, which may be secondary to the fact that those targets exhibited very low homologies between dog and human and therefore could not be recognized.

High titer inhibitory antibodies to IFNγ have been previously described to cause susceptibility to intracellular pathogens, such as mycobacteria, salmonella, CMV, VZV and certain filamentous fungi, in certain ethnic backgrounds. However low titer, de facto non-inhibitory antibodies may be more widely found (Doffinger Lab, unpublished observation). We found antibodies to IFNγ, both in patients with ID and in patients with AIDs. Interestingly all of our ID patients with anti IFNγ antibodies (8 out of 18) presented with infection due to obligate or facultative intracellular pathogens including fungi (n = 4), viruses (n = 3) and *Salmonella* (n = 1) (Fig. 3). We found anti IFNγ antibodies as well in AID patients (9 out of 26) which interestingly mainly present with SLE (4). Several studies suggest a role of IFNγ in the induction of autoimmunity and particularly in the etiology of systemic lupus (Domeier et al., 2016; Green et al., 2017). High titer, neutralizing antibodies to IL-6 have been described in patients with severe bacterial infections (Puel et al., 2008; Nanki et al., 2013). We did not identify significant anti- IL-6 autoantibodies in the ID group, but instead in 3 dogs with AID presenting with SLE, RA, and MG. IL-6 plays a key role in inflammation by activating the acute phase response and by potentially causing B-cell hyperactivity (Narazaki et al., 2017; Wallace et al., 2017), and it has been shown to be involved in the pathogenesis of various autoimmune diseases including RA (Narazaki et al., 2017) and SLE (Tackey et al., 2004). A recent report showed that treatment with the IL-6R antagonist Tocilizumab was beneficial in a patient with MG (Jonsson et al., 2017). Increased levels of antibodies to IL-6 have been previously noted in patients with autoimmune disease and an exacerbating role was suggested (Takemura et al., 1992).

4.2. Higher complexity of anti-cytokine responses in patients with AID

AID patients (n = 32) displayed more complex anti-cytokine responses with 27% showing high positivity to more than 3 cytokines versus only 6.25% in the ID group. Mainly targeted cytokines include TH17 cytokines (IL-17A/F, IL-21, IL-22; IL-23; n = 18), the TH1 cytokine IFNγ (n = 9) and the regulatory cytokine IL-10 (n = 9). The balance between TH17 cells/cytokines and Treg cells with IL-10 as one signature cytokine is thought to play an important role in the induction of autoimmune diseases (Noack and Miossec, 2014). The high incidence of ACAB´s in AID patient may reflect the particular induction of their targets and thus be considered as 'reactive' and regulative or it may play a more direct part in the etiology of the AID condition either by exacerbating pro-inflammatory cytokines (e.g. Th17 and Th1) or by attenuating the effect of regulatory factors (e.g. IL-10).

In this context, it is particular noteworthy that two IBD patients (n = 4) presented with autoantibodies to IL-10 and two as well with antibodies to the Th17 cytokines IL-17A and IL-22. Genetic defects in the IL-10 signaling pathway have been shown to cause IBD (Glocker et al., 2011). Therapeutic blockade of IL-17A has recently been associated with various forms of gastrointestinal inflammation including IBD in several recent report (Wang et al., 2018; Vernero et al., 2019).

Patients with ulcerative keratitis (UK) and epidermolysis bullosa acquisita (EBA) exhibited autoantibodies to IL-17 and/or IL-10 (UK: 4 out of 5; EBA: 3 out of 3). Th17 responses are reported to play key roles in both UK and EBA. Therapeutic TH17 blockade of IL-17A are considered as a possible treatment options (Cao et al., 2017; Castela et al., 2019).

4.3. Th1/Th17 cytokines and IL-10 are main targets in patients with infectious diseases

Interestingly all of our ID patients with anti-IFNγ antibodies (8 out of 18) presented with infection due to obligate or facultative intracellular pathogens including fungi (n = 4), viruses (n = 3) and *Salmonella* (n = 1). We identified anti TH17 antibodies in 7 out of 16 dogs with ID, presenting with fungal (5) and bacterial infections (2). These findings are in line with previously reported susceptibilities in the human system (Browne, 2014; Barcenas-Morales et al., 2019). Anti-IL-10 autoantibodies were identified in 7 dogs with ID. IL-10 is an antagonist of IFNγ and an important regulatory component in the response to infection helping to find the correct balance between antimicrobial efficiency and host injury (Couper et al., 2008). Both inhibitory and enhancing antibodies could be considered to play a role in the regulation of infection but also may have detrimental effects. In summary, data suggest disease specific anti-cytokine response pattern. This is in line with previously published data in humans (Rosenberg et al., 2016).

4.4. Rare anti-cytokine specificities

Interestingly, the only two patients found to have antibodies to IL-21 were both presenting with myositis. A recent report suggested a link between autoimmune myositis and high levels of IL-21 (Kageyama et al., 2017). One patient with lymphoma showed an unusual anti-cytokine response pattern including autoantibodies to GM-CSF and G-CSF. Antibodies to IFNβ were only identified in one patient presenting with IBD. IFNβ administration has been shown to induce clinical remission in a subset of patients with ulcerative colitis (Mannon et al., 2011).

5. Conclusion

In this study, we show a significant presence of anti-cytokine autoantibodies in sera of canines with various immune related diseases including recurrent infections, autoimmune diseases and cancer in comparison to healthy controls. In addition, we show distinct associations of various disease types to the specificity of anti-cytokine autoantibodies and to the number of targeted cytokines. Results are likely to be relevant for the wider field of mammalian immunology. The wide occurrence of ACAB strongly suggests, that they have immuno-regulatory functions and reflect the induction of distinct sets of cytokines in infection and inflammation. A more direct involvement in the various disease pathologies may be considered but will need in detail functional studies, which was not the aim of the current study. The investigation of anti-cytokine autoantibodies may give us new insights into immune disease mechanisms and states of immune dysregulation and may allow us to develop new tools for disease diagnosis and intervention. Further studies with larger patient numbers will be needed to ascertain the association ACAB's with the various types of autoimmunity and susceptibility to infectious disease.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2019.109902.

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