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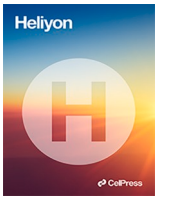

Research article

CD163, a novel receptor for TNF, was revealed *in situ* by proximity ligation assay☆

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ABSTRACT

Cytokine therapy utilizes cytokines to enhance the immune system to fight diseases. These strategies rely on advanced knowledge, including the communication between cytokines and their receptors. *In situ,* cytokine-receptor interactions are typically analyzed by co-localization using immunolabeling. Our study compared co-localization using the Proximity Ligation Assay (PLA), a recently developed *in situ* protein-protein interaction technique. In an inflamed porcine lung model, we demonstrated the efficacy of PLA in detecting interactions between tumor necrosis factor (TNF) and its receptors TNFR1 and TNFR2. Additionally, the CD163 receptor was identified as a novel partner of TNF. Furthermore, the combination of immunolabeling and PLA offered additional insights, particularly, the internalization of TNF following its binding with CD163 in macrophages. Our work focused on *in situ* interactions of TNF with macrophages TNF receptors and suggested exciting perspectives for further understanding and application of cytokine-based therapies.

1. Introduction

Cytokines are signaling molecules essential in cell communication within the immune system and beyond [1]. Cytokine therapy involves the therapeutic use of various cytokines or their inhibitors to regulate the immune system and treat various diseases [2–4]. This approach holds great promise for regenerative medicine [3,5,6]. Tumor Necrosis Factor (TNF) inhibitors or anti-TNF drugs, have revolutionized the treatment of immune-mediated inflammatory diseases affecting the gut, joints, skin, and eyes by modulating inflammation [7,8]. Anti-TNF therapies, including monoclonal antibodies and soluble receptors, suppress the physiological response to

☆ **One-sentence summary:** By using a combination of PLA and immunolabeling for the first time, we demonstrated that TNF interacted with the CD163 receptor on macrophage cells.

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TNF, though they can also have side effects [9]. A deeper understanding of interactions between cytokines and their receptors could pave the way for developing new targeted therapeutic molecules.

TNF is a multifunctional cytokine that plays a role in several biological processes: i) as an inflammatory cytokine, TNF plays a

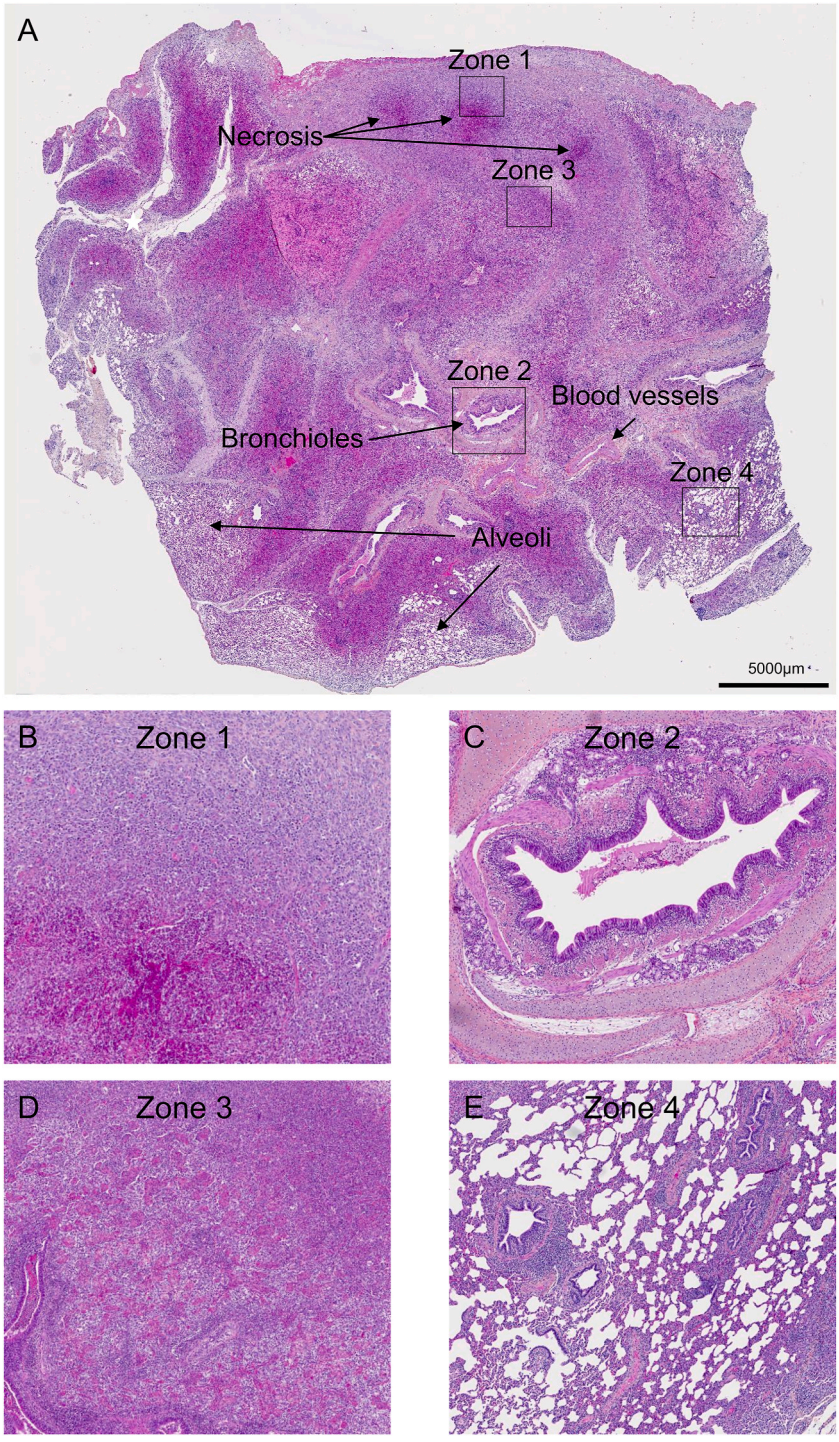


Fig. 1. Macroscopic morphology of inflamed lung tissue of PIG-4. (**A-E**) HPS-stained lung sections. (**B-E**) Magnified view: higher magnification images of lung morphology from four representative areas within the black rectangle of image A. (**B**) Zone 1, tissue around the necrosis; (**C**) Zone 2, inflamed bronchioles; (**D**) Zone 3, inflamed tissue where the native histological lung structure was not detectable; and (**E**) Zone 4, tissue still containing alveoli lung structure.

crucial role in directly triggering inflammatory responses to injury or infection. It also indirectly promotes inflammation by inducing cell death [7,10,11]. ii) In certain contexts, and at specific concentrations, TNF contributes to tissue repair and regeneration [12]. iii) TNF affects the proliferation and differentiation of progenitor cells and immune cells [13]. iv) It plays a vital role in angiogenesis [14]. v) TNF exhibits anticancer activity [15]. vi) TNF neutralization has been identified as a potential therapeutic approach to modulate tissue regeneration in various diseases and injuries [8,16,17]. However, the duality of TNF's effects on both inflammation and regeneration presents a challenge in developing targeted therapies [7].

The TNF receptor superfamily (TNFRSF) is a group of cytokine receptors characterized by their ability to bind TNF [18–20]. Among the TNFRSF, Tumor necrosis factor receptor 1 (TNFR1), is widely distributed and mediates various cellular responses, activating both inflammatory and apoptotic pathways [11,21,22]. TNFR2 is another important receptor expressed in more restricted cell types (immune cells, endothelial cells, nerve cells, and Mesenchymal Stem Cells) compared to TNFR1 [23]. TNF binding to TNFR2 triggers specific signaling cascades distinct from those activated by TNFR1 [10]. The functions of TNFR2 are often complementary to those of TNFR1, but its signal transduction is directed toward promoting cell survival, proliferation, and immune regulation rather than inducing inflammation and apoptosis. TNFR2 has attracted attention for its potential therapeutic applications in promoting tissue regeneration and immune regulation [23].

Similar to TNF, Tumor necrosis factor-like weak apoptosis inducer (TWEAK), is a member of the TNF superfamily that controls a multitude of cellular processes including proliferation, migration, differentiation, apoptosis, angiogenesis, and inflammation [24]. In vitro, it interacts with CD163 [25,26], a scavenger receptor with anti-inflammatory properties, primarily expressed in macrophages and monocytes [27–29]. CD163-expressing macrophages can bind and internalize TWEAK protein added exogenously from the supernatant [25,30].

The interaction between a cytokine and its receptor can be investigated through protein-protein interactions *in situ* using a co-localization approach. Several experimental techniques are commonly employed: i) Bioluminescence- and Förster resonance energy transfer are as sensitive *in situ* methods for detecting molecular interactions, but both techniques require a high level of expertise to ensure specificity [31]. ii) Bimolecular fluorescence complementation is a technology typically used to validate protein interactions. However, this method which relies on recombinant fluorescent protein fragments has some limitations [32]. iii) Immunolabeling is a useful technique for detecting the proximity of two molecules within a cellular compartment, though it does not indicate a direct binding between them [33–35]. iv) Proximity Ligation Assay (PLA), based on oligonucleotide-labeled antibody pairs and rolling circle amplification, offers advantages over conventional methods. This versatile technique enhances sensitivity and accuracy by significantly amplifying the signal-to-noise ratio, allowing for quantitative analysis of protein-protein interactions in cells and tissues [36–40].

Our objective was to explore the capacity of the innovative PLA approach to identify cytokine-receptor interactions *in situ,* within an inflamed pig lung tissue. Since tissue regeneration in pigs closely resembles that in humans, pigs serve as an excellent model for studying inflammation and tissue regeneration [41–46]. We focused on the interaction between TNF and two TNFRSF (TNFR1 and TNFR2) along with the CD163 scavenger receptor, using CD206 (C-type lectin receptor) protein as a negative control of binding. Both CD163 and CD206 are receptors found on the surface of certain immune cells, particularly macrophages [47]. *In situ* detection of TNF interaction with these three receptors confirmed the efficacy of the PLA technique for studying protein-protein interactions *in situ.* Based on our experiments, PLA proves to be a sensitive method for investigating the complexity of the resolution of inflammation *in situ*.

2. Results

2.1. Inflammation-induced structural damage in the lung tissue

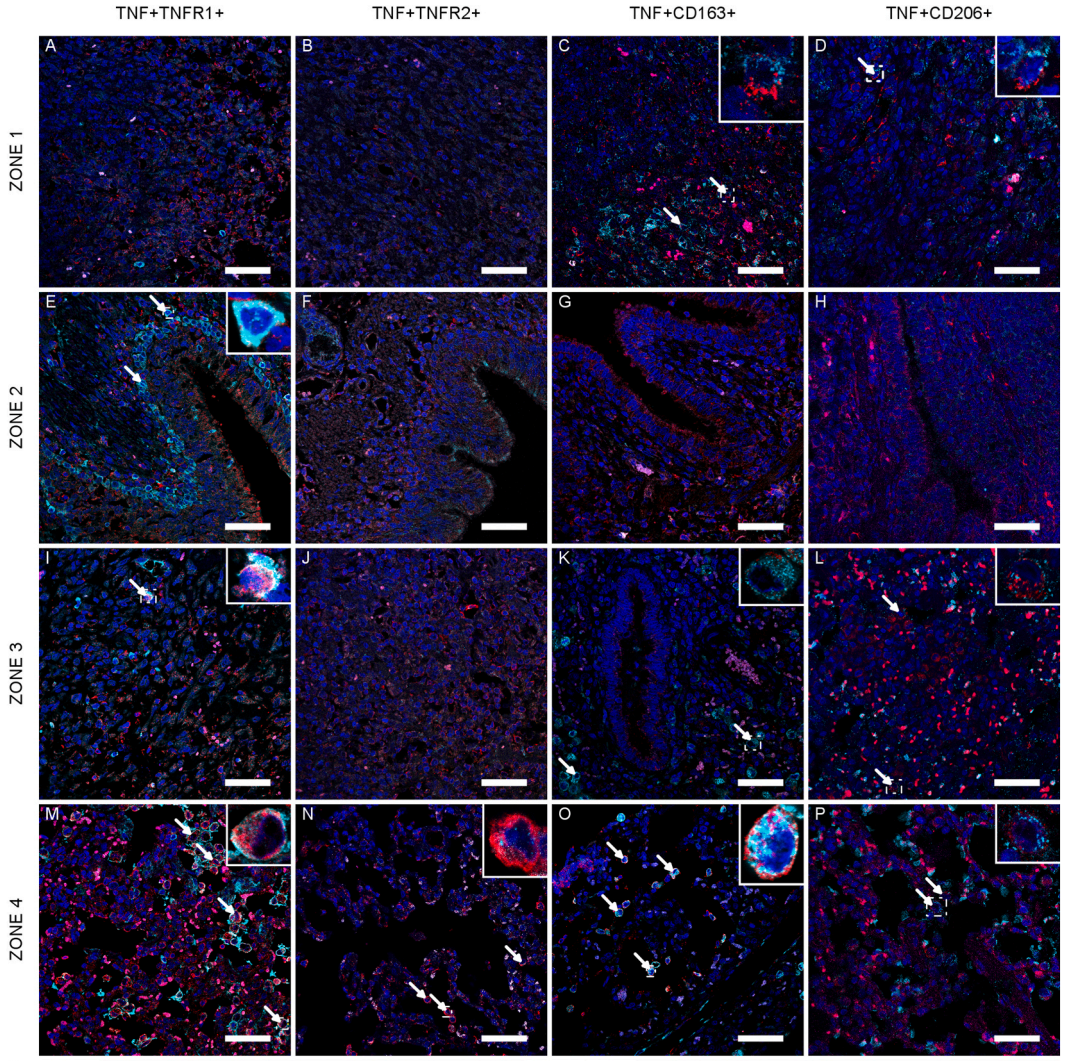
Histological staining was used to visualize the morphology and analyze the structure of lung tissues collected from one healthy pig (PIG-1), two pigs with spontaneously developed pneumonia (PIG-2, PIG-3), and one injured pig exposed to organophosphorus (PIG-4) (Fig. S1). Our ethical approval complies with ARRIVE guidelines. Representative image from three independent labeling experiments is presented. Compared to the healthy lung (Fig. S1A), the inflamed lungs (Figs. S1B–S1D) displayed notable differences, including an increase in inflammatory cells and activation of endothelial cells, indicating the presence of inflammation. Both PIG-2 and PIG-3 exhibited less severe inflammation (Figs. S1B and S1C), allowing visualization of the alveoli after staining. In contrast, the PIG-4 showed severe inflammation with lung necrosis (Figure S1D and Fig. 1). For further experiments, four different areas were selected from the most inflamed lung tissue (PIG-4) (Fig. 1A) comprising: Zone 1, featuring necrosis (Fig. 1B); Zone 2, inflamed bronchiolar region (Fig. 1C); Zone 3, inflamed tissue with macrophages but lacking alveolar structures (Fig. 1D); zone 4, a less disordered region with preserved alveoli lung structures (Fig. 1E).

2.2. TNF expression screening and antibody specificity

The initial step focused on indirectly assessing TNF expression in the inflamed lung samples, by detecting the expression of TNF Alpha Induced Protein 1 (TNFAIP1) through immunohistochemistry (IHC). Due to the limited availability of pig-specific antibodies, human antibodies are commonly used to analyze porcine proteins. TNFAIP1 showed a high degree of amino acid sequence homology (98 %) between human and porcine variants (Fig. S2A). In the negative control, the absence of primary antibodies resulted in no labeling (Fig. S3A). Immunohistochemical staining with anti-TNFAIP1 was successful in both PIG-2 and PIG-3 (data not shown), while a strong signal was detected in the most inflamed lung tissue (PIG-4) (Fig. S3B), indicating a significant TNFAIP1 expression *in situ*.

The next step involved testing suitable antibodies on PIG-4 lung tissue against targeted partners TNFR1, TNFR2, CD163, and CD206. Two anti-TNFR1 antibodies were evaluated: anti-TNFR1-(1) and anti-TNFR1-(2). The anti-TNFR1-(1) antibody showed no specific labeling (Fig. S3C), while anti-TNFR1-(2) displayed a strong labeling (Fig. S3D). The anti-TNFR2 antibody did not yield any observable labeling (Fig. S3E). In contrast, the anti-CD163 antibody indicates effective detection of CD163 in the tissue (Fig. S3F).

Despite an 87 % amino acid similarity between human and porcine TNF proteins (Fig. S2B), the next phase involved assessing three



Q

	TNF+	TNFR1+	TNF+TNFR1+	TNF+TNFR1+ /TNFR1+
Zone 1	64.80	14.80	13.10	88.50
Zone 2	69.30	37.10	33.60	90.56
Zone 3	61.10	22.30	20.70	92.80
Zone 4	69.20	26.90	24.30	90.33

R

	TNF+	TNFR2+	TNF+TNFR2+	TNF+TNFR2+ /TNFR2+
Zone 1	54.50	1.00	0.60	60.00
Zone 2	73.80	1.50	0.80	53.30
Zone 3	53.50	1.30	0.80	53.80
Zone 4	62.70	10.60	7.20	67.90

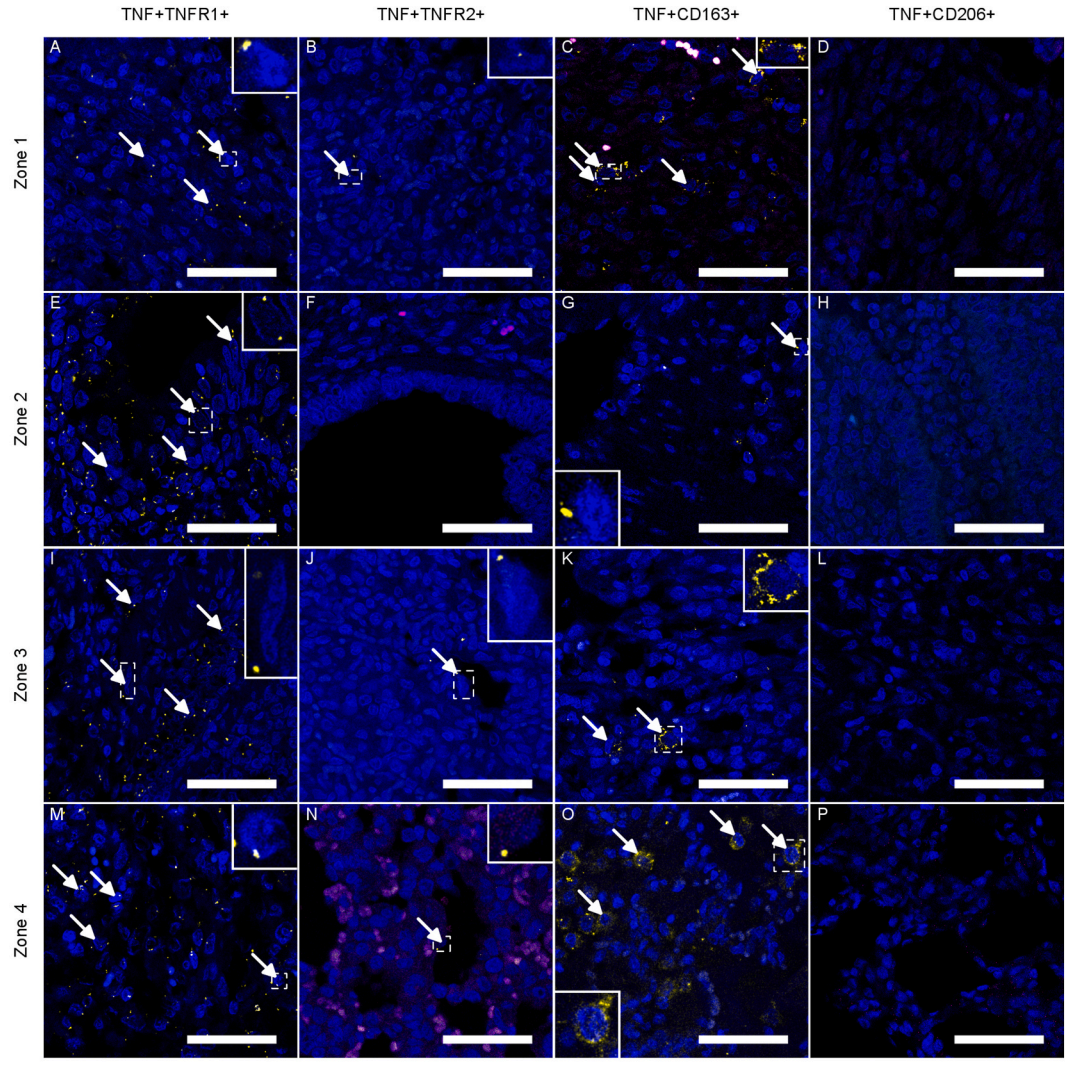
S

	TNF+	CD163+	TNF+CD163+	TNF+CD163+ /CD163+
Zone 1	71.90	34.30	32.40	94.50
Zone 2	71.80	1.60	1.30	84.20
Zone 3	52.60	8.50	8.10	95.30
Zone 4	42.70	16.30	12.50	76.70

T

	TNF+	CD206+	TNF+CD206+	TNF+CD206+ /CD206+
Zone 1	45.50	32.30	22.50	69.60
Zone 2	54.10	5.40	4.70	84.80
Zone 3	44.80	25.80	20.50	79.30
Zone 4	45.70	19.90	16.00	80.50

Fig. 2. Co-localization of TNF and four receptors in the inflamed lung zones *in situ.* (**A,E,I,M**) Immunolabeling of both TNF and TNFR1; (**B,F,J,N**) both TNF and TNFR2; (**C,G,K,O**) both TNF and CD163 and (**D,H,L,P**) both TNF and CD206. (**A-D**) Zone 1, (**E-H**) Zone 2; (**I-L**) Zone 3, and (**M-P**) Zone 4. Quantitative analysis of the expression of (**Q**) TNF and TNFR1, (**R**) TNF and TNFR2, (**S**) TNF and CD163, (**T**) TNF and CD206 in the different regions. Quantitative analysis based on random examination of 3 sets of 1000 cells per condition. Percentage of colocalization. Anti-TNFR1, anti-TNFR2, anti-CD163, anti-CD206 (Alexa Fluor 488, cyan), anti-TNF (Alexa Fluor 568, red fluorescence). Nuclear staining with DAPI (blue fluorescence). Autofluorescence of the blood red cells (magenta). Scale bar = 50 μm. Arrow: co-localization of the immunolabeling.



Q

Zone	Method	TNF+TNFR1+	TNF+TNFR2+	TNF+CD163+	TNF+CD206+
Zone 1 (%)	Colocation	13.1	0.6	**32.4**	**22.5**
	PPI	11.5	0.6	**13.1**	**0.1**
Zone 2 (%)	Colocation	33.6	0.8	1.3	**4.7**
	PPI	39.2	2.8	1.5	**0.1**
Zone 3 (%)	Colocation	20.7	0.8	8.1	**20.5**
	PPI	17.8	1.4	9.4	**0.1**
Zone 4 (%)	Colocation	24.3	7.2	12.5	**16.0**
	PPI	30.5	2.7	8.3	**0.1**

Fig. 3. Interaction between different cytokines and receptors *in situ*. (**A,E,I,M**) Proximity Ligation Assay with TNF and TNFR1; (**B,F,J,N**) with TNF and TNFR2; (**C,G,K,O**) with TNF and CD163 and (**D,H,L,P**) with TNF and CD206. (**A-D**) Zone 1, (**E-H**) Zone 2; (**I-L**) Zone 3, and (**M-P**) Zone 4. (**Q**) Quantitative analysis of the interaction between cytokine and receptors in the different regions. Quantitative analysis based on random examination of 3 sets of 1000 cells per condition. Percentage of interactions, in red significant difference between Co-localization and IPP quantification ($p < 0.05$, *t*-test). Cytokine/receptor interaction in yellow. Nuclear staining with DAPI (blue fluorescence). Autofluorescence of the blood red cells (magenta). Scale bar = 50 μm. Arrow: interaction between cytokine and receptor.

anti-human TNF antibodies against porcine TNF (Figs. S3G–S3I). Detecting cytokines such as TNF is challenging due to their secretion, which leads to dilution in the extracellular matrix. Among anti-TNF-(1), -(2), and -(3) antibodies, anti-TNF-(1) was selected for further analysis as it generated a specific signal significantly faster than the other two antibodies. Anti-TNF labeling took 30 s (1), 1 min (2), and 5 min (3) to reach approximately the same intensity (Figs. S3G–S3I).

Regarding CD206 detection, the specific anti-CD206 antibody revealed significant protein expression in inflamed pig lung cells (Fig. S3J).

To enhance the binding affinity of antibodies, an antibody epitope retrieval approach was employed by breaking down protein cross-links formed during tissue fixation. The commonly heat-induced epitope retrieval (HIER) method particularly with citrate buffer (pH 6), is often effective. However, in our IHC experiments, this method did not result in detectable labeling of TNFR2 protein (Fig. S3E). In a subsequent experiment, HIER was performed using Tris-EDTA buffer (pH 9) (Fig. S4), which successfully produced satisfactory labeling with the anti-TNFR2 antibody (Fig. S4E). This buffer was subsequently adopted for all immunolabeling experiments.

In summary, antibody specificity was screened via IHC. Epitope retrieval using Tris-EDTA buffer (pH 9) was validated and proven effective for all antibodies, including anti-TNFR2, and was thus selected for ongoing experimentation.

2.3. Co-localization of cytokines and receptors in the inflamed lung tissue of PIG-4

Following antibody validation was investigated. The co-localizations of TNF/TNFR1, TNF/TNFR2, TNF/CD163, and TNF/CD206 were studied using immunofluorescence in the four lung zones (Fig. 2A–E,I,M; 2B,F,J,N; 2C,G,K,O and 2D,H,L,P, respectively). Cyan indicates the expression of different receptors, while red represents the presence of TNF. Cells positive for both cytokines and receptors are identified by overlapping red and cyan signals. DAPI (4′,6-diamidino-2-phenylindole) is an intercalating molecule that binds strongly to DNA and is commonly used in cell biology and microscopy to visualize cell nuclei, which appear blue when stained with DAPI.

In zone 1, TNF displayed a moderate co-localization rate with TNFR1, and a weak co-localization rate with TNFR2 (Fig. 2A and B) whereas TNF showed a stronger co-localization signal with the CD163 and CD206 receptors in this region (Fig. 2C and D).

In zone 2, only TNF/TNFR1 co-localization was prominent in the sub-epithelial region (Fig. 2E), while co-localization of TNF/TNFR2, TNF/CD163, and TNF/CD206 was negligible (Fig. 2F-H).

In zone 3, TNF/TNFR1 exhibited the strongest co-localization intensity (Fig. 2I), TNF/CD163 and TNF/CD206 proteins displayed significantly reduced levels, while the TNF/TNFR2 protein pairs demonstrated very low co-localization levels (Fig. 2J and L).

Zone 4 was the region where all four protein pairs demonstrated considerable co-localization (Fig. 2M–P) whereas no signal was observed in the negative controls (absence of primary antibodies) (Fig. S5). An additional negative control was included in the experiment. Despite using antibodies with different species-specificities, there is a possibility of nonspecific antibody binding due to a high degree of amino acid sequence homology. To assess this, we used an anti-human Glial Fibrillary Acid Protein (GFAP) antibody, along with the same secondary antibody for receptor detection. This negative control confirmed that no nonspecific binding was associated with the antibody isotype (Fig. S5E). The highest level was observed for TNF/TNFR1 (Fig. 2M), followed by TNF/CD163 (Fig. 2O), and TNF/CD206 (Fig. 2P), with the lowest level observed for co-localization between TNF and TNFR2 proteins (Fig. 2N).

To confirm our observations, TNF, TNFR1, TNFR2, CD163, and CD206 positive cells were quantified and the percentage of co-localization cells was estimated for 1000 cells across the four zones (Fig. 2Q). It is important to note that due to the high secretion and dilution of TNF in the extracellular space, quantifying the number of cells expressing TNF presents challenges. A variance of 20–50 % may occur during quantification. TNF levels were elevated in all four zones, while in the TNF/TNFR1, TNF/CD163, and TNF/CD206 samples, the amount of free TNF receptors was very low, ranging from 4.7 % to 30.4 %. Interestingly, the free TNFR2 levels were higher in the TNF/TNFR2 samples. TNF/TNFR1 co-localization was significantly higher in zone 2, whereas TNF/TNFR2 co-localization was higher in zone 4. Regarding TNF/CD163 co-localization, zone 1 contained the highest percentage of positive co-localized cells around 32 %. Finally, the biological state of tissue injury is a critical factor to consider before screening co-localized proteins. In all cases, the cytokine-positive cells were more abundant than receptor-positive cells. The percentage of receptor binding a cytokine was 88 %–92 % for TNFR1, 53 %–67 % for TNFR2, 76 %–95 % for CD163, and 69 %–84 % for CD206.

2.4. In situ protein-protein interaction of cytokines and receptors in the inflamed lung tissue of PIG-4

The co-localization analysis was the first approach to improve our understanding of protein-protein interactions (PPI), and the results were confirmed using a specific PPI technique *in situ*. The PLA technique was performed to investigate the interaction between cytokines and TNFR1, TNFR2, and CD163 receptors within the four selected lung zones (Fig. 3). The yellow color represents the interaction between the different receptors and TNF, while DAPI-stained nuclei are shown in blue. No PPI was detected in the negative control (absence of the primary antibody) (Fig. S6). The TNF and CD206 pair were selected as a negative control of PPI (Fig. 3D–H,L, and P).

The PPI profile was similar to the co-localization quantification result (Fig. 3A–B, 3E-G, 3I-K, 3M – O and 3Q). However, the interaction between TNF and CD163 receptor (Fig. 3C and Q) displayed a significant difference in the initial conditions, with much lower PPI in cells than anticipated based on the co-localization results (Fig. 3Q). No interaction was observed between TNF and the second macrophage-specific receptor CD206, despite their intense co-localization (Fig. 3D–H,L, and P).

In conclusion, the PLA method effectively detected protein-protein interactions, successfully identifying CD163 as a novel TNF receptor.

2.5. The association of co-localization and PPI underscores the potential internalization of the TNF/CD163 interaction in macrophages

The combination of immunolabeling and PLA not only provides valuable insights into the spatial and functional aspects of protein interactions within cells but also facilitates the identification of the specific cellular environment where these interactions occur. TNF/CD163 interaction was investigated to determine whether the interacting proteins are located on the macrophage surface or undergo internalization. Iba1 (ionized calcium-binding adaptor molecule 1) has emerged as a key membrane marker, widely recognized for its efficiency in identifying macrophages in various tissues, including the lung [48]. Iba1 immunolabeling was integrated to examine the localization of TNF/CD163 interaction in zone 4. Iba1 marks of macrophages (cyan color). The yellow color indicates TNF/CD163 interaction, while nuclei stained with DAPI are blue. Cells displaying all three colors indicate macrophages where a protein-protein interaction between TNF and the CD163 receptor has been formed. A comparative IPP of TNF/CD163 (Fig. 4A) in combination with Iba1 (Fig. 4B; Video 1) showed a predominant interaction within macrophages. There was no interaction between TNF and the CD206 receptor in macrophages (Fig. 4C), nor in the negative control (Fig. 4D). This combined approach not only highlighted the cellular localization of the interaction, specifically its occurrence in macrophages but also detailed the exact location of the interaction. While the expression of Iba1 was localized at the plasma membrane, the interaction itself was largely internalized (Video 1). Thus, using these two techniques provides a more delicate nuanced understanding of the cellular interaction process.

2.6. In vitro protein-protein interaction between TNF and CD163

Proximity ligation assay (PLA) is a technique that can detect protein-protein interactions within a spatial range of approximately 40 nm. While this method identifies proteins in close proximity, it does not definitively confirm direct interactions. To validate the interaction between TNF and CD163 observed in our PLA experiments, we employed surface plasmon resonance spectroscopy (SPR) to investigate the interaction between TNF and CD163 in real-time *in vitro*. Due to the unavailability of purified recombinant porcine proteins, these experiments were conducted on mouse proteins. SPR analysis confirmed the interaction between mouse TNF and mouse TNFR1, which served as a positive control of binding (Table 1). Additionally, mouse CD163 demonstrated binding to immobilized mouse TNF in the presence of calcium. To further characterize the interaction between mouse TNF and mouse CD163, decreasing

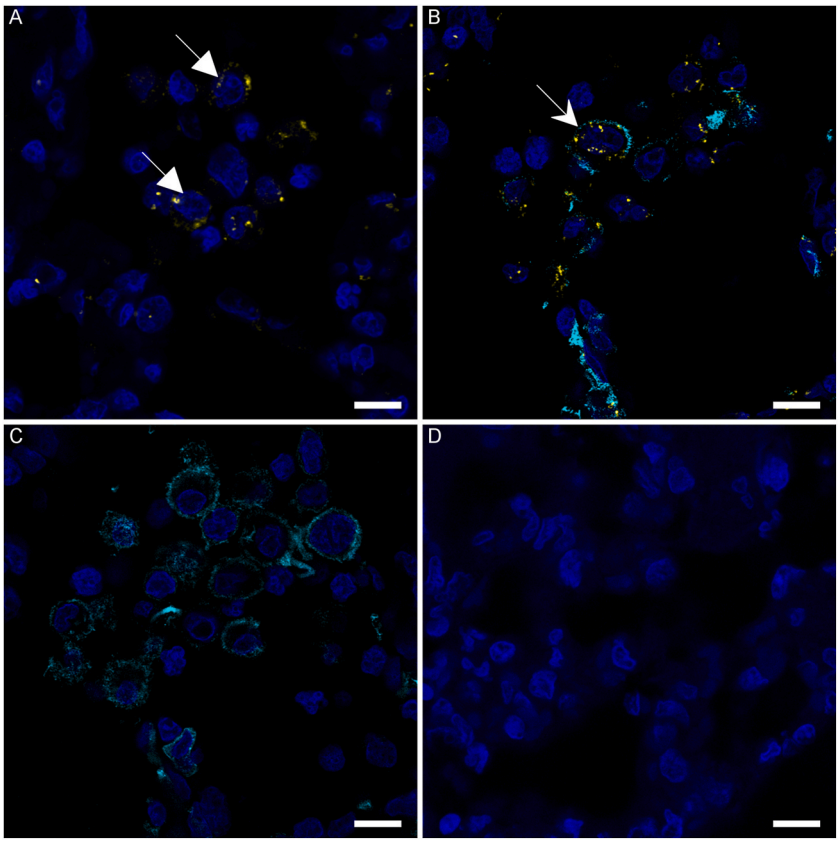


Fig. 4. Combination of immunolabeling with IPP. (A) IPP between TNF and CD163 proteins. (**B**) IPP between TNF/CD163 and immunolabeling with anti-Iba1 antibody. (**C**) IPP between TNF/CD206 and immunolabeling with anti-Iba1 antibody. (**D**) Negative control for IPP between TNF/CD163, IPP between TNF/CD206, and immunolabeling with anti-Iba1 antibody. Cytokine/receptor interaction in yellow. Anti-Iba1 (Alexa Fluor 488, cyan). Nuclear staining with DAPI (blue fluorescence). Scale bar = 10 μm. Arrow flat: PPI of the TNF/CD163. Arrow: Iba1 immunolabeling.

Table 1
Kinetic and dissociation constants for the binding of CD163 and TNFR1 to immobilized mouse TNF.

Immobilized Ligand	Soluble Analyte	Ka (M^{-1} s^{-1})	Kd (S^{-1})	K_D (M)	Chi2
Mouse TNF	Mouse TNFR1	5.45×10^{3}	$3{,}79 \times 10^{-4}$	6.96×10^{-8}	0.24
Mouse TNF	Mouse CD163	2.5×10^{6}	2.32×10^{-4}	9.3×10^{-11}	0.165
Mouse TNF	Human CD163	7.85×10^{4}	1.51×10^{-4}	1.93×10^{-9}	0.634

amounts of CD163 were injected over the immobilized mouse TNF. As shown in figure SFig. 7, binding was dose-dependent, and further kinetic analysis of the binding data yielded association and dissociation rate-constants of 2.5×10^{6} $M^{-1}s^{-1}$ and 2.32×10^{-4} $M^{-1}s^{-1}$, respectively, using a global fitting to a 1:1 Langmuir interaction model (Table 1). The deduced apparent equilibrium dissociation constant (KD) was 6.96×10^{-8} nM, indicating a high affinity between mouse CD163 and mouse TNF.

Human CD163 interacted with mouse TNF (data not shown). Similarly, the interaction between human TNF and both human TNFR1 and TNFR2 was assayed, and a specific interaction was observed (data not shown).

3. Discussion

Cytokine therapy involves the use of cytokines to modulate the immune system for treating various diseases. Despite its success, the efficacy of cytokine therapy can be inconsistent [3,5,6]. Employing novel techniques to explore and comprehend interactions between cytokines and their receptors is crucial for optimizing cytokine-based therapies and ultimately improving patient outcomes.

Currently, only a limited number of tools are available for the *in situ* analysis of cytokine-receptor interactions. One commonly used method is the study of protein co-localization [49–51]. Although it provides valuable insights, our research has underscored the limitations of this traditional method. We contrasted it with the PLA technique demonstrating its advantages. The detection of cytokine-receptor interactions relies on two key factors: first, the recognition of the protein which depends on selecting the appropriate antibodies; and second factors such as taking into account the expression level of the analyzed protein, cytokine secretion level, and the protein half-life [52].

The second factor is the number of cytokine receptor partners.

As evaluated using the PLA technique, the interaction between TNF and its receptor may, in some cases, show differences compared to co-localization results. In zone 1, near the area of necrosis, the interaction between CD163 receptor and TNF is significantly smaller compared to the co-localization results. This discrepancy may also suggest that the receptor in this region may have a function other than interacting with TNF, though further analysis is needed to confirm this.

This result suggests that co-localization results may suffice when cytokine has a single receptor and protein detection is straightforward. However, observing co-localization alone may not be sufficient to draw definitive conclusions in cases where cytokines bind to several distinct receptors. In this context, the PLA approach was proposed as a valuable alternative.

Moving on to TWEAK and TNF, both members of the TNF superfamily, these proteins interact with different receptors and signaling pathways. TWEAK targets Fn14 (fibroblast growth factor-inducible 14), while TNF interacts with TNFR1 and TNFR2 receptors, triggering specific cellular responses [8]. [53] CD163 primarily functions as a receptor involved in the clearing of hemoglobin-haptoglobin complexes and has anti-inflammatory properties [29,54,55]. Although CD163 can interact with various ligands, including TWEAK, its interaction with TNF is uncertain [53]. In this study, the interaction between TNF and CD163 we specifically investigated. Initially, co-localization studies provided insight into cellular distribution. However, we used the PLA technique to confirm protein interactions, thus presenting for the first time evidence of an interaction between CD163 and TNF. Understanding which cytokine interacts with which receptor is the first step in understanding the complex processes involved in inflammation and regeneration. To gain deeper insight into cell-cell interactions, it is essential to identify the specific cells involved in these interactions. Different cells may produce or respond to the same cytokine in various ways, affecting the overall outcome of the immune response [1–6]. Therefore, in addition to mapping cytokine-receptor pairs, a comprehensive understanding of inflammatory and regenerative processes requires knowledge of the precise cellular context in which these interactions occur. To determine whether the interaction occurred within macrophages, we used a combined approach that integrated immunolabeling and PLA within the same tissue section. Iba1, a macrophage marker, was used for immunolabeling, while antibodies against TNF and CD163 were used to test cytokine-receptor interaction [56]. This approach provides valuable insight into the spatial and functional aspects of protein interactions within cells, precisely defining the specific cellular environment in which these interactions occur. In addition, the internalization of the receptor-ligand complex into the cell was also an important phenomenon. In our study, we specifically investigated the TNF/CD163 interaction within macrophages, focusing on whether the interacting proteins remained on the cell surface or were internalized. The combination of the two methods showed that, in contrast to TNFR1 and TNFR2 receptors, CD163 is highly internalized by TNF.

A decoy receptor is a specialized receptor that binds to signaling molecules, such as cytokines, without transmitting a signal. By preventing these molecules from interacting with their functional receptors, decoy receptors inhibit their effects and play an important role in regulating inflammation [57].

Decoy receptors are part of a distinct subgroup within the TNF receptor superfamily (TNFRSF) and play a critical regulatory role in modulating cellular responses to TNF superfamily ligands [58]. Unlike signaling receptors, decoy receptors lack intracellular signaling domains or possess non-functional signaling regions, enabling them to bind ligands without initiating downstream signaling cascades. This competitive binding helps sequester ligands away from functional receptors, thereby dampening pro-inflammatory, apoptotic, or

survival signals depending on the context. Decoy receptors are essential in maintaining homeostasis, preventing excessive immune activation, and protecting tissues from inappropriate or prolonged responses to TNF superfamily ligands.

Several decoy receptors have been identified within the tumor necrosis factor (TNF) superfamily. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has emerged as a promising candidate for cancer therapy. However, many cancers are resistant to TRAIL-induced apoptosis [59–61]. TRAIL decoy receptors, including decoy receptor 1 (TRAIL-R3), decoy receptor 2 (TRAIL-R4), and osteoprotegerin (OPG), play critical roles in modulating TRAIL-related signaling. These receptors bind to TRAIL without activating downstream apoptotic pathways, acting as molecular "decoys" to regulate cellular responses and induce TRAIL resistance [61,62].

The potential existence of a decoy receptor for TNF remains an area of significant interest. The soluble form of TNFR2 is thought to act as a potential decoy receptor for TNF, but conclusive evidence confirming the existence of a true decoy receptor for TNF is still lacking [63]. If such a receptor were to be identified, it could significantly influence the regulation of TNF's biological effects, potentially reducing its pro-inflammatory and cytotoxic actions in pathological conditions. Identifying a decoy receptor for TNF could reveal new regulatory mechanisms and therapeutic targets within TNF signaling pathways.

Watany and colleagues (2024) demonstrated that CD163 functions as a decoy receptor for TWEAK [64]. Our results suggest that CD163 can be a potential decoy receptor for TNF also (Fig. 5, Fig. S7 and Table 1). Unlike conventional TNF receptors, which actively propagate signaling cascades, CD163 may function by binding TNF and sequestering it from interacting with signaling receptors, thereby blunting its pro-inflammatory effects. This hypothesis is supported by the role of CD163 in the resolution of inflammation, as it is upregulated in response to anti-inflammatory stimuli and plays a key role in the clearance of inflammatory mediators.

Given the central role of TNF in triggering inflammation in diseases such as rheumatoid arthritis and Crohn's disease, the possibility of CD163 acting as a decoy receptor introduces a new regulatory mechanism that can be targeted by anti-inflammatory therapies [65]. [66] Further research is needed to confirm the ability of CD163 to modulate TNF activity and its broader effects on immune regulation.

The SPR analysis confirms the interaction between TNF and CD163 in a mouse model. This finding supports our *in situ* PLA study in porcine lung tissue, which suggested a close spatial relationship between these proteins. The use of SPR provided direct evidence of this interaction, reinforcing our confidence in the PLA observations despite the species difference. This result suggests a conserved interaction between species (pig TNF/pigCD163 using PLA, mouse TNF/mouse CD163 using SPR, and mouse TNF/human CD163 using SPR), highlighting a biological function of TNF interaction for CD163.

In summary, our research highlights the advantages of the PLA technique, offering novel insights into cytokine-receptor interactions and cell-cell communication that were previously inaccessible. These findings enhance our understanding of the complex interactions between TNF and its receptors during inflammation and regeneration. The interaction between TNF and TNFR1 plays a role in the initiation of inflammation and/or apoptosis. The precise involvement of TNFR2 requires further investigation. However, the interaction between TNF and CD163 appears promising as a key factor in reducing inflammation and promoting regeneration. We hypothesize that CD163 interacts with TNF, facilitating its internalization, thereby removing it from the extracellular matrix and potentially playing a crucial role in regeneration processes.

Our work in the context of *in situ* interactions and porcine models offers exciting prospects for advancing the understanding and application of cytokine-based therapies in the pursuit of enhanced tissue regeneration.

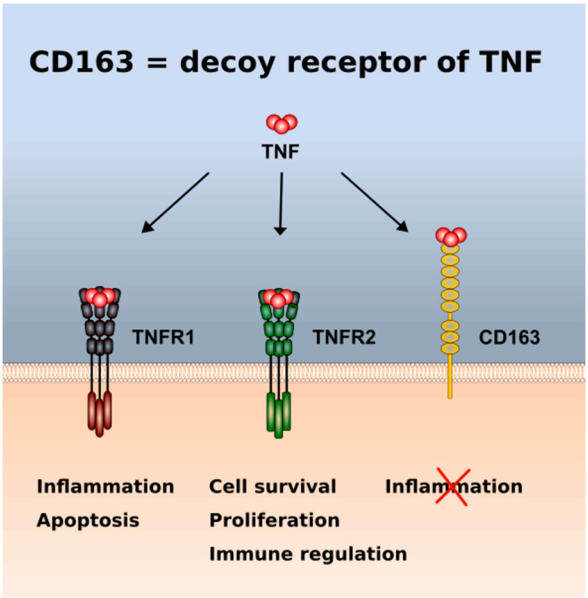


Fig. 5. Our findings indicate that CD163 may serve as a decoy receptor for TNF, potentially modulating inflammation by binding to TNF or related ligands without initiating downstream signaling. By acting as a decoy, CD163 could block TNF from engaging with its functional receptors, thereby dampening inflammatory responses.

4. Limitations of the study

A potential limitation of this study results from the capacity of the PLA technique to identify only a single protein interaction per sample, preventing from exploring the complexity of these interactions. In the future, new methods should be developed to thoroughly investigate the complexity of cytokine-receptor interactions.

CRediT authorship contribution statement

Alexandre Cousin: Investigation. **Myriam Oger:** Writing – original draft, Visualization. **Aymar de Jenlis:** Validation, Investigation. **Audrey Lejart:** Validation, Investigation. **Laure Barbier:** Investigation. **Diane Riccobono:** Writing – review & editing. **Xavier Holy:** Writing – review & editing. **Anne-Laure Favier:** Writing – original draft, Supervision, Conceptualization. **Krisztina Nikovics:** Writing – original draft, Validation, Supervision, Methodology, Conceptualization.

Data availability statement

Data associated with the study has not been deposited into a publicly available repository and data will be made available on request.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Diane Riccobono reports financial support was provided by Work was supported by French Defence Central Health Service and the General Delegation for Armement (DGA) (PDH2-NRBC-4-NR-4306). If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2025.e42194.

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