contribute to the disease process. While stimulation of TDAG8 in immune cells may dampen the inflammatory process, stimulation of OGR1 and GPR4 may increase inflammation and fibrosis, at least in the case of increased expression of OGR1 and GPR4 in inflamed tissue, possibly also driven by hypoxia. Although human genetics studies demonstrate an important role for TDAG8 in IBD, the interplay between the three proton-activated receptors needs further attention to understand their respective roles.

As hypoxia, lactate and other metabolites and acidosis emerge not only as key characteristics of inflamed tissue but also as local modulators of the immune response, their relative contributions and mutual interactions remain to be further dissected. Clearly, all three are linked by molecular and cellular events, but their specific effect on distinct immune cells and also on the response of resident cells, such as epithelial cells, fibroblasts and vascular cells, is only just emerging, and a better understanding of these events may offer exciting insights into processes of inflammation and tissue repair. ❐

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Competing interests

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Check for updates

STRUCTURAL IMMUNOLOGY

Overcoming the LAG3 phase problem

Lymphocyte activation gene 3 (LAG3) is an important checkpoint inhibitor molecule of immunotherapeutic interest. New crystal structures of LAG3 provide important insight into its molecular architecture, laying the groundwork for future basic and applied investigations.

Jan Petersen and Jamie Rossjohn

Basic discoveries regarding immune checkpoint molecules, such as CTLA4 and PD1, have enabled a greater understanding of the 'molecular brakes' that regulate T cell reactivity (Allison and Honjo, 2018 Nobel Prize in Physiology or Medicine). These findings have led to the development of cancer immunotherapies that target these checkpoint inhibitors[1]. One lesser-understood checkpoint inhibitor, LAG3, has proven a difficult molecule to investigate. In this issue of *Nature Immunology*, Ming et al.[2] provide fundamental insight into the molecular architecture of the LAG3 ectodomain, helping to unlock the wider biology and immunotherapeutic potential of this molecule.

In the modern era of structural biology, it is relatively rare for an immunoreceptor of interest to remain uncharacterized structurally. Take, for example, the impressive rapidity with which the structures of the spike protein of the SARS-CoV-2 virus, either alone or bound by protective monoclonal antibodies, have been elucidated[3]. However, some molecules, for a variety of reasons, can remain intransigent to study and require a series of approaches (such as protein engineering) before the 'molecular nut' is cracked. Indeed, LAG3 was first identified in 1990 (ref. [4]), yet atomic information about this molecule has been lacking until now. The innovation in the study by Ming et al.[2] was to use a yeast display library approach to select variants of LAG3 that express at higher levels and higher affinity to a ligand when compared to that of wild-type LAG3. This opened the door for structural and biophysical studies of this molecule.

Why go to such lengths to study LAG3 structurally? A primary function of LAG3 is the negative regulation of major histocompatibility complex class II (MHCII)-mediated T cell activation (reviewed elsewhere[5]). Much like its structural ancestor, the CD4 co-receptor, with which it shares low sequence homology (20%), LAG3 associates with the αβ T cell receptor (TCR) on the cell surface. When TCRs engage MHCII molecules on antigen-presenting cells, LAG3 also binds to MHCII molecules and negatively regulates TCR signaling by driving co-receptor–Lck dissociation[6]. Presumably, LAG3 operates similarly to CD4 in that it binds to MHCII alongside the TCR and its signaling apparatus. However, in contrast to CD4, which enhances TCR signaling, LAG3 somehow acts to disband the signaling complex[7]. LAG3 distribution and clustering on the cell surface therefore affects its activity. This is where LAG3 biology gets convoluted, as, in addition to MHCII, five other ligands of LAG3 have been discovered, namely fibrinogen-like protein 1 (FGL1), LSECtin, galectin 3 and α-synuclein in association with amyloid beta precursor-like protein 1 (APLP1)[5,8]. Although LAG3 is expressed across the gamut of T cells in a TCR signaling-dependent manner, its presence and activity on immune cells that lack TCR expression (natural killer cells, B cells and plasmacytoid dendritic cells) indicates that LAG3 might have multiple functions, with its multi-ligand interactions presumably providing some contextual adaptability.

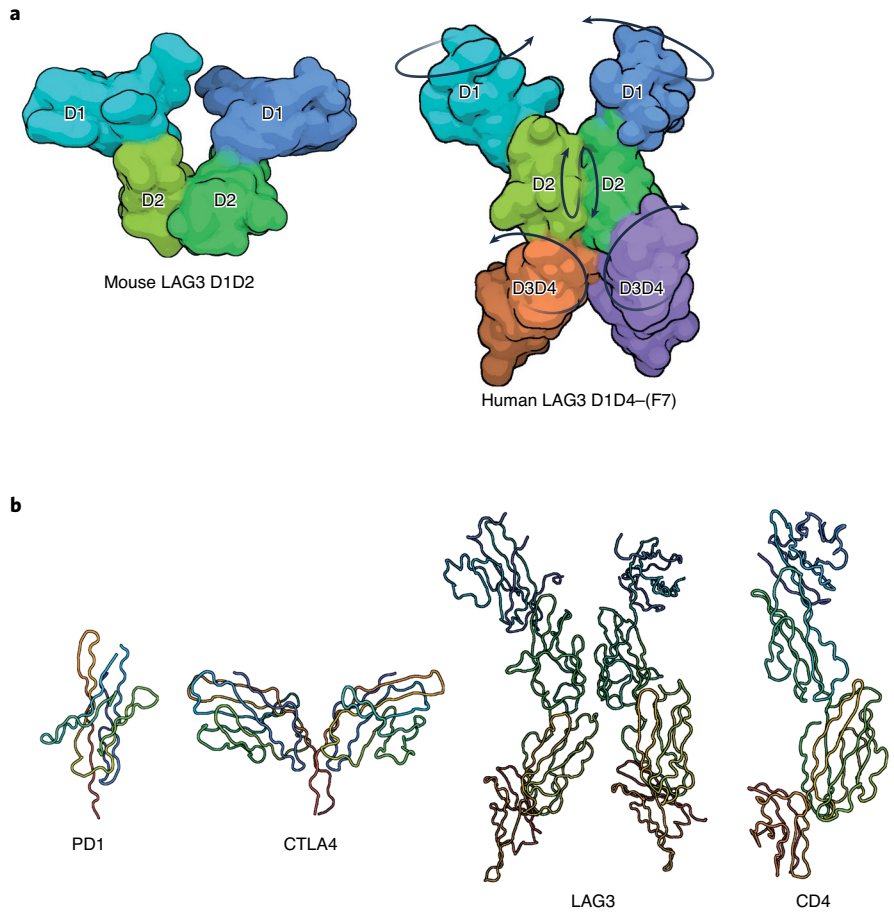

Fig. 1 | LAG3 structure, flexibility and comparison to co-stimulatory molecules and co-receptors. a, Comparison of the crystal structures of mouse LAG3 dimer including domains D1 and D2 (left), and human LAG3 dimer including domains D1–D4 (right) from the LAG3 complex structure with F7 scFv (not shown). Protein surface representations are coloured according to observed positional variability of domains (D1, D2 and D3D4), with arrows indicating presumed inter-domain flexibility of human LAG3 D1D4. **b**, Structures of T cell co-stimulatory molecules PD1, CTLA4, LAG3 and CD4 shown as tube representations. Figure created with BioRender.com.

Although a structural report (no coordinates deposited in the Protein Data Bank) of the LAG3 domain 1 in complex with the Fab fragment of ieramilimab (a LAG3 inhibitor) has been published[9], the current study provides the first molecular detail of the entire (D1–D4) ectodomain of this enigmatic regulator protein. To identify the LAG3 ectodomain structure, the authors co-crystallized LAG3 D1–D4 with a single-chain variable fragment (scFv) of the F7 antagonist. Furthermore, to enable building of the LAG3 structure, they also characterized the structures of the more stable and biochemically better-behaved mouse LAG3 D1D2 and the human LAG3 D3D4 fragment in complex with the F7 scFv (Fig. 1).

So what does the structure tell us? Although the complete LAG3 ectodomain structure is admittedly of low resolution, it was sufficient to show that LAG3 formed a homodimer via the D2 domain, with the remaining domains forming an elongated, but curved arrangement. The dimer interface (verified by mutagenesis) in D2 was angled, so that the D1 domains were tilted away from the central axis of the dimer and formed a V-shaped aperture. Whereas the dimer interface in the human LAG3 structure and mouse LAG structure used a broadly conserved set of residues, the angle formed by the D1D2 dimer was surprisingly different (23.9° versus 69.6°) and would result in distinct relative positions of the presumed MHCII- and FGL1-binding sites. In the human LAG3 structure, the potential MHCII-binding loop 1 of the D1 domain (also named 'extra loop' for its absence in the ancestral homolog CD4) was rotated about 90° away from the central axis, whereas it was oriented towards the aperture in the mouse D1D2 structure. It is possible that these conformational differences between human and mouse LAG3 provide snapshots into two functionally distinct states of LAG3 and the switch could be enabled by considerable plasticity in the dimeric arrangement. The D3 and D4 domains of the LAG3 were interconnected by a substantial interface. Intriguingly, the presence of the inhibitory F7 scFv in each monomer of the LAG3 D1D4–F7 structure forced one of the D3D4 domains to flip into a different orientation relative to D1D2. Whether the inhibitory activity of F7 is due to a structural disturbance in the juxtamembrane of the D4 domains or rather a result of this disturbance causing a switch in D1 is unclear. Indeed, several features of the LAG3 structures, including interdomain flexibility and conformational variability within loops that mediate binding to physiological ligands and monoclonal antibodies, suggest that the dynamics of the LAG3 structure has a mechanistic role in its biology.

In addition to providing structural information on the LAG3 ectodomain, Ming et al.[2] also solved the structure of FGL1 and mapped the interface between LAG3 and FGL1 using yeast display screening. This structure identified crucial interface residues in loop 2 in D1 of LAG3, which coincided with the site of an antagonist antibody, and demonstrated that LAG3 binds MHCII and FGL1 via distinct molecular surfaces. Together with the FGL1 structure, the interface map led to the conclusion that the LAG3 dimer could not reasonably form a 2:2 complex with the dimeric FGL1. This conclusion is intriguing, as it provides a glimpse of how FGL1 might alter TCR signal regulation by LAG3 (that is, by taking the brakes off a T cell). By clustering LAG3 together on the cell surface, FGL1 could reversibly modulate LAG3 activity without direct interference in MHCII binding. This structural information on LAG3 and FGL1 provides some crucial cornerstones that should help to decipher the mysterious modus operandi and interactions of LAG3. Indeed, many questions remain, particularly regarding the structural basis of LAG3 interactions with its various ligands (including HLA II) and why distinct MHCII allomorphs exhibit differing affinities for LAG3[7].

A string of biotech companies are investing in LAG3 programs, and 115 clinical trials related to LAG3 are presently listed in the ClinicalTrials.gov database. The structural work presented here not only provides insight into LAG3 biology, but also serves as a basis for assessing agonist and antagonist antibodies that target LAG3, and the rational development of small-molecule inhibitors that target LAG3–ligand interactions. Although LAG3 structural and

functional work may have lagged behind other checkpoint inhibitors, a series of important studies in this axis[6,7,10,11], including from Ming et al.[2], represent critical steps in our understanding of LAG3 structure and function. ❐

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Competing interests

The authors declare that they have a research collaboration with Immutep, a company exploring the use of targeting LAG3 in cancer immunotherapy.

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CANCER IMMUNOLOGY

Deciphering immunoregulatory vulnerabilities in human cancers

Regulatory T cells that express high levels of IL-1R and ICOS display transcriptional features of antigen specificity, are highly suppressive and distinguish tumors from non-malignant inflamed tissues

Felipe Gálvez-Cancino, Alvaro Lladser and Sergio A. Quezada

In the past decade, immunotherapy has revolutionized oncological treatments through the development of monoclonal antibodies designed to block inhibitory signaling pathways that normally restrict anti-tumor T cell immunity. These regulatory pathways are also known as immune checkpoints and their blocking antibodies as checkpoint inhibitors. Although checkpoint inhibitors can re-ignite T cell activity in tumors, they can also promote re-activation of T cells that infiltrate inflamed non-malignant tissues, promoting on-target, off-tumor adverse side effects in a notable proportion of patients and occasionally leading to patient death[1]. In addition, adverse events result in immediate cessation of treatment, which can, in many cases, lead to tumor relapse. Hence, the identification of molecular pathways that are specifically active in tumors but absent in non-malignant inflammatory sites could hold the key to the development of improved immunotherapies with maximal anti-tumor activity and minimal toxicity.

In a recent issue of *Nature*, Mair et al.[2] address this challenge by comparing, at the single-cell level and using unsupervised bioinformatic tools, the immune landscape of human head and neck squamous cell carcinoma (HNSCC) tumors and matched, non-malignant, inflamed oral mucosal tissues (Fig. 1). The authors found a well-conserved immune landscape across malignant and non-malignant inflamed tissues, including the presence of previously described tissue-resident and exhausted T cells that express high levels of the immune checkpoint molecule PD-1. However, a few key differences were highlighted by their high-dimensional flow cytometry data coupled to a machine-learning analysis tool developed to discover and annotate statistically relevant cellular phenotypes[3]. These included tumor-enriched CD4$^+$ regulatory T (T_{reg}) cell populations marked primarily by the expression of ICOS, and a population of conventional dendritic cells (cDCs) displaying activation markers, including CD40 and PD-L1.

To gather a deeper understanding of the immune microenvironment in HNSCC, the authors performed single-cell RNA sequencing in more than 140,000 cells from 8 different patients[2]. Complementing the cytometry data, transcriptionally altered type 1 and type 3 conventional dendritic cells (cDC1s and cDC3s) expressing *TGFB1*, *IL18BP* and *ICOSLG* and specific transcriptional profiles in CD8$^+$ and T_{reg} cells highlighted a potential cross-talk between innate and adaptive immune compartments. Using NicheNet[4], a tool that predicts cellular communications between receptors and ligands, the authors identified several potential interactions between myeloid cells and T_{reg} cells driven by ICOS ligand and ICOS, IL-18 and IL-18R1, and IL-1B and IL-1R1. Furthermore, this population of IL-1R1–ICOS-expressing T_{reg} cells was clonally expanded and characterized by a transcriptional signature consistent with recent T cell antigen receptor (TCR) engagement, which suggests that these cells may be interacting with cDC1 and cDC3 in an antigen-specific manner. These results are consistent with previous findings for melanoma and other tumor types in which the clonality of tumor-infiltrating T_{reg} cells has been associated with their ability to recognize tumor antigens[5]. Although these data support a specific interaction between the antigen-presenting cell and T_{reg} cell compartment in HNSCC tumors, the direct or indirect effects of cDC1, cDC3 and T_{reg} cell subsets on CD8$^+$ T cell priming and effector function remain less explored. To use similar tools to identify key pathways that control such interactions may offer further insights in the regulation of anti-tumor T cell responses, and equally important, may provide potential targets for therapeutic evaluation.

In addition to being phenotypically different from T_{reg} cells infiltrating non-malignant tissues, IL-1R1$^+$ICOS$^+$ T_{reg} cells demonstrated high suppressive activity of effector CD4$^+$ and CD8$^+$ T cell proliferation in vitro, supporting a potentially relevant role of these cells in the control of anti-tumor immunity. Of relevance, these findings are not limited