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Interleukin-1-abhängige Produktion von Interleukin-22 in Brust- und Lungenkarzinom-Zelllinien und Interleukin-37-induzierte Modulation der angeborenen Immunantwort

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2. Verzeichnis der Abkürzungen

γδ T-Zellen	gamma delta T-Zellen
AP-1	*Activator protein 1*
AhR	*Aryl hydrocarbon receptor*
BD2	β-Defensin 2
CANTOS	*Canakinumab Anti-inflammatory Thrombosis Outcomes Study*
ELISA	*Enzyme-linked immunosorbent assay*
FACS	*Fluorescence-activated cell sorting*
FGF	*Fibroblast growth factor*
HBE	*Primary human bronchial epithelial cells*
HEK	*Human embryonic kidney cells*
HUVEC	*Human umbilical vein endothelial cells*
IL	Interleukin
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
IL-1Ra	IL-1-Rezeptor-Antagonist
IL-10R2	IL-10 Rezeptor 2
IL-18Rα	IL-18 Rezeptor α
IL-18BP	IL-18-bindendes Protein
IL-22BP; IL-22RA2	IL-22-bindendes Protein
IL-22R1; IL-22RA1	IL-22 Rezeptor 1
ILC	*Innate lymphoid cells*
kDa	Kilodalton
LCN2	Lipocalin 2
LPS	Lipopolysaccharid
MALT	Mucosa-assoziiertes lymphatisches Gewebe

MAPK	*Mitogen-activated protein kinase*
MDSC	*Myeloid-derived suppressor cells*
MM	Malignes Melanom
MYD88	*Myeloid differentiation primary response 88*
NF-kB	*Nuclear factor kappa B*
NKT	Natürliche-Killer-T-Zellen
NLRP 3, 6	*NLR family pyrin domain containing 3, 6*
PBMC	*Peripheral blood mononuclear cells*
PI3K-Akt-mTOR	*Phosphatidylinositide 3-kinase-Akt-mammalian target of rapamycin*
PNAS	*Proceedings of the National Academy of Sciences of the USA*
pro-IL-1α	IL-1α-Vorläuferpeptid
pro-IL-1β	IL-1β-Vorläuferpeptid
RORγt	*RAR-related orphan receptor gamma*
ROS	*Reactive oxygen species*
SAA	Serum Amyloid A
SMAD3	*SMAD family member* 3
STAT 1, 3, 5	*Signal transducers and activators of transcription 1, 3, 5*
TAM	Tumor-assoziierte Makrophagen
T_c17	Typ17 zytotoxische T-Zelle
T_h1	Typ1-T-Helferzelle
T_h17	Typ17-T-Helferzelle
T_h22	Typ22-T-Helferzelle
TGF-β	*Transforming growth factor-β*
TLR	*Toll-like receptors*
Treg	Regulatorische T-Zelle
VEGF	*Vascular endothelial growth factor*
Wnt/β-Catenin	*Wingless and INT-1/* β*-Catenin*

3. Veröffentlichungen

Die Ergebnisse dieser Arbeit wurden in folgenden internationalen Fachzeitschriften als Originalarbeiten veröffentlicht:

1. Voigt C*, May P*, **Gottschlich A***, Markota A, Wenk D, Gerlach I, Voigt S, Stathopoulos GT, Arendt K, Heise C, Rataj F, Janssen KP, Königshoff M, Winter H, Himsl I, Thasler WE, Schnurr M, Rothenfußer S, Endres S, Kobold S (*contributed equally).
   Cancer cells induce interleukin-22 production form memory CD4+ T cells via interleukin-1 to promote tumor growth.
   Proceedings of the National Academy of Sciences of the United States of America 2017; 114(49):12994-12999.

2. Eisenmesser Z. E, **Gottschlich A**, Redzic J, Paukovich N, Azam T, Zhang L, Zhao R, Jeffrey S. Kieft, The E, Meng X, Dinarello A. C
   The interleukin-37 monomer is the active form for reducing innate immunity
   Proceedings of the National Academy of Sciences of the United States of America 2019;116(12):5514-5522.

4. Einleitung

4.1 Inflammation und Tumorentwicklung

Der fundamentale Zusammenhang zwischen entzündlichen Prozessen und Tumorerkrankungen konnte schon im 19. Jahrhundert festgestellt werden. 1863 beschrieb Rudolf Virchow leukozytäre Infiltrate in neoplastischem Gewebe und hypothesierte ein komplexes Netzwerk zwischen diesen auf den ersten Blick gänzlich unterschiedlichen Krankheitsprozessen (Balkwill and Mantovani 2001). Seither wurden chronische Entzündungsprozesse als Risikofaktoren für eine Vielzahl von unterschiedlichen Tumorentitäten identifiziert. Tabelle 1 gibt eine Übersicht über einige wichtige Neoplasien und die korrelierenden inflammatorischen Prozesse.

Tabelle 1: Assoziation zwischen Inflammation und Tumorerkrankungen
(modifiziert nach Balkwill und Mantovani 2001).

Tumorerkrankung	Entzündlicher Stimulus oder Grunderkrankung
Blasenkarzinom	Schistosomen
Zervixkarzinom	Papillomavirus
Magenkarzinom	Helicobacter pylori-assoziierte Gastritis
MALT Lymphom	Helicobacter pylori-assoziierte Gastritis
Hepatozelluläres Karzinom	Hepatitis-B-Virus, Hepatitis-C-Virus
Kolorektales Karzinom	Chronisch entzündliche Darmerkrankungen
Bronchialkarzinom	Nikotinabusus, Asbestose und Silikose

Chronische Entzündungsreaktionen, wie sie z. B. im Rahmen der Autoimmunerkrankungen Morbus Crohn und Colitis ulcerosa auftreten, sind je nach Schweregrad und Manifestation der Erkrankung mit einer erhöhten Inzidenz von kolorektalen Karzinomen (CRC) assoziiert. Generell ist von einer um ein-einhalb bis zweifach erhöhten Inzidenz für die Entwicklung eines CRC im Vergleich zur durchschnittlichen Bevölkerung auszugehen. Bei schwerer Manifestationsform, beispielsweise im Sinne einer Pancolitis, kann das Lebenszeitrisiko für die Entwicklung eines CRC sogar bis zu 15% übersteigen (Beaugerie and Itzkowitz 2015, Beaugerie, et al. 2013). Darüber hinaus ist die mit Nikotinabusus assoziierte chronische Entzündung der Bronchien einer der Hauptrisikofaktoren für die Entwicklung von Bronchialkarzinomen. Bis zu 30% aller Krebserkrankungen werden durch Nikotinabusus und inhalierte Noxen ausgelöst (Aggarwal, et al. 2009). Erst 2017 konnten im Rahmen der multizentrischen *Canakinumab Anti-inflammatory Thrombosis Outcomes Study* (CANTOS) klinische Hinweise gesammelt werden, dass die Gabe eines Interleukin-1β (IL-1β)-blockierenden

Antikörpers die Inzidenz von Lungenkarzinomen reduzieren könnte (Ridker, et al. 2017). Diese CANTOS Studie hebt die Relevanz und die Aktualität des komplexen Zusammenspiels zwischen Entzündungsprozessen und Tumorentwicklung hervor und zeigt, dass weitere Forschungsbemühungen zum besseren Verständnis dieser komplexen Netzwerke nötig sind.

4.1.1 Grundlagen der Entzündungskaskade

Schon in den 1980er Jahren waren die Gemeinsamkeiten zwischen Tumorgewebe und Wunden nachgewiesen und Karzinome wurden als „*Wounds that do not heal*“ beschrieben (Dvorak 1986). Um die zentrale Rolle von Entzündungsvorgängen auf die Entwicklung und Progression von Tumorerkrankungen besser verstehen zu können, soll im Folgendem zunächst kurz auf die nach Gewebsverletzungen stattfindenden physiologischen Reparaturprozesse eingegangen werden. Das Verständnis dieser Prozesse ist essentiell, da viele der genannten Prozesse auch im Rahmen von malignen Erkrankungen wiederzufinden sind.

Im Rahmen von Gewebstraumata kommt es zunächst zu einer Thrombozytenaggregation und zur Bildung eines Gewebethrombus. Die aktivierten Thrombozyten sezernieren vasoaktive Stoffe, wie beispielsweise Arachidonsäure-Metabolite und Serotonin, die eine erhöhte Gefäßpermeabilität und einen erhöhten Einstrom von Fibrinogen bedingen. Als Resultat kommt es zur Bildung eines fibrinogenhaltigen Thrombus. Chemotaktische Faktoren wie *transforming growth factor-β* (TGF-β) und *platelet-derived growth* factor freigesetzt durch die aktivierten Thrombozyten tragen zur Bildung von Granulationsgewebe und zur Aktivierung von Fibroblasten bei. Im Rahmen dieser Signalkaskade kommt es außerdem zur Freisetzung von proteolytisch wirksamen Enzymen, welche zu einer Remodellierung der extrazellulären Matrix beitragen. Durch weitere chemotaktische Signale kommt es zu einer Rekrutierung von Granulozyten, Monozyten und Fibroblasten und zur Restaurierung der verletzen Gefäße. Der reziproke Signalaustausch zwischen Immunzellen und Zellen stromaler und epithelialer Herkunft führt zu einer Re-Epithelialisierung des verletzten Gewebes und im Idealfall zu einer *Restitutio ad integrum* (Coussens and Werb 2002).

Viele der hier beschrieben Mechanismen können auch in neoplastischem Gewebe beobachtet werden, wobei das empfindliche Gleichgewicht zwischen pro- und anti-inflammatorischen Prozessen häufig gestört ist (vgl. Abbildung 1).

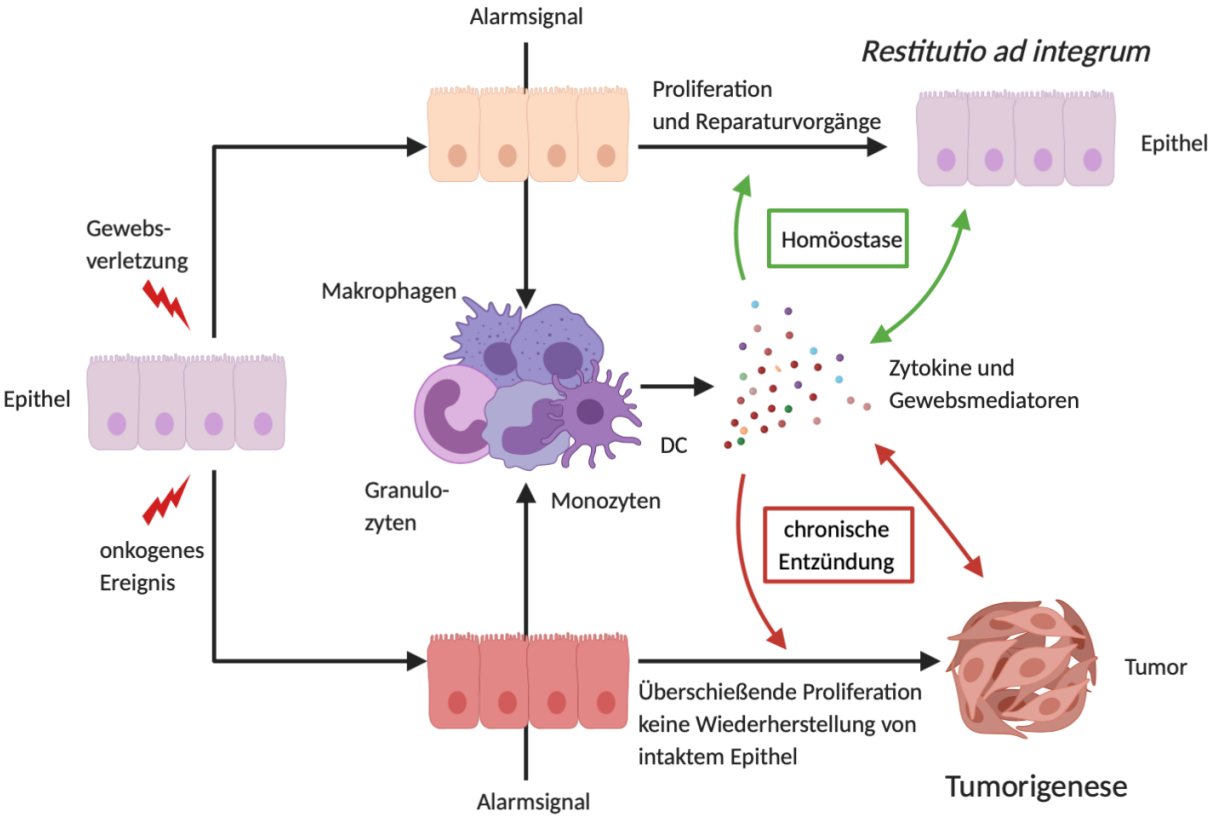

Abbildung 1: Gemeinsamkeiten und Unterschiede in der Entwicklung von Inflammation und Neoplasien (modifiziert nach Greten and Grivennikov 2019).

4.1.2 Aufbau und Organisationsprozesse maligner Erkrankungen

Getrieben durch die enormen Forschungsbemühungen der vergangenen Jahrzehnte hat sich das Verständnis der Tumorbiologie entscheidend gewandelt. Mit dem Bestreben, die komplexen biologischen Veränderungen in einem Tumor zu gliedern, propagierten *Hanahan und Weinberg* im Jahr 2000 in ihrer wegweisenden Veröffentlichung sechs „*Hallmarks of Cancer*“: (1) Aktivierung proliferativer Signalkaskaden, (2) Umgehung der Wachstumsinhibition, (3) aktivierende Invasions- und Metastasierungsprozesse, (4) unerschöpfliche Teilungsfähigkeit (Immortalisierung), (5) Induktion der Angiogenese und (6) Evasion von Apoptose-mechanismen (vgl. Abbildung 2, Hanahan and Weinberg 2000).

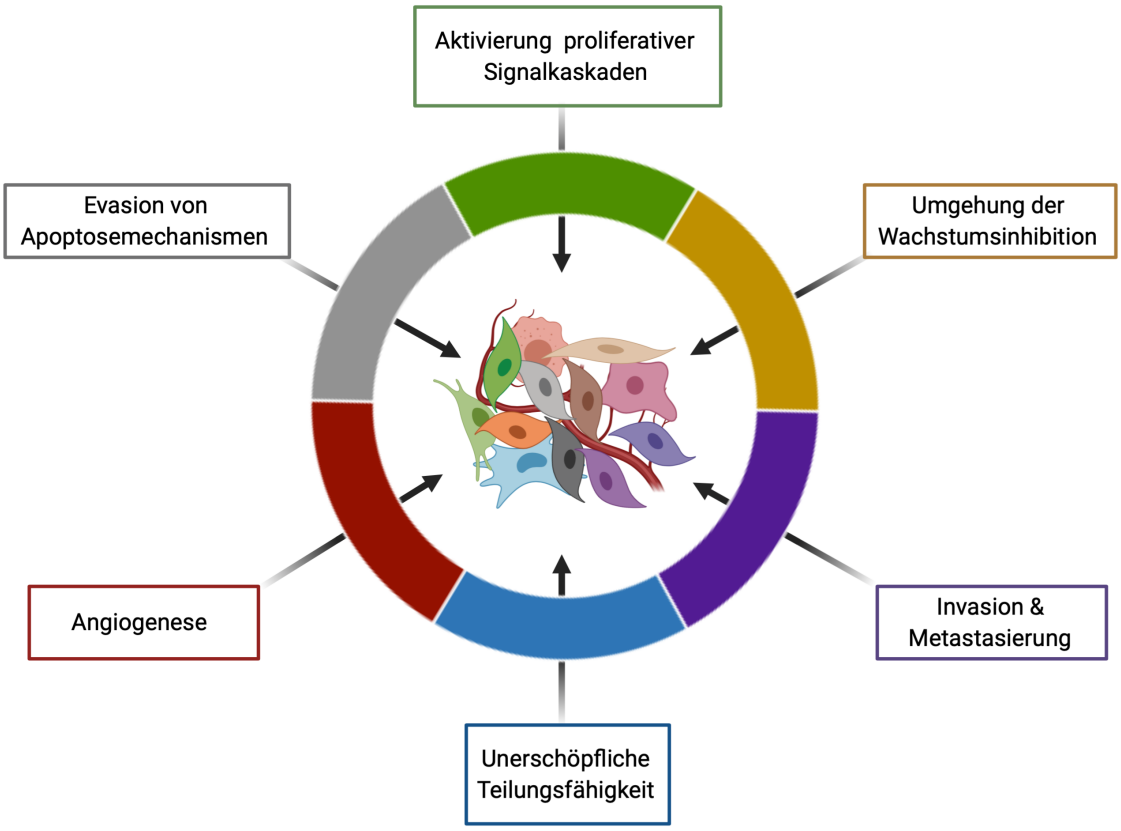

Abbildung 2: *Hallmarks of Cancer* (modifiziert nach Hanahan and Weinberg 2000).

Diese „*Hallmarks of Cancer*“ wurden 2011 nochmals durch weitere fundamentale Charaktereigenschaften von Neoplasien ergänzt (vgl. Abbildung 3, Hanahan and Weinberg 2011). Entzündungsprozesse (*„tumor-promoting Inflammation“)* können auf unterschiedlichen Ebenen zum Erwerb der oben beschriebenen Eigenschaften beitragen. Die Sekretion von biologisch aktiven Molekülen führt zu einer Aktivierung proliferativer Signalkaskaden und zur Inhibition von Zelltodmechanismen. Darüber hinaus führen die ausgeschütteten Zytokine und Chemokine zu einer Verstärkung der Angiogenese und zu einer Remodellierung der extrazellulären Matrix, mit einer daraus resultierenden gesteigerten Fähigkeit der Metastasierung. Weiterhin besitzen durch Immunzellen freigesetzte chemische Botenstoffe wie beispielsweise *reactive oxygen species* (ROS) mutagene Eigenschaften, welche durch genetische Evolution der Tumorzellen zu einer erhöhten Malignität führen können.

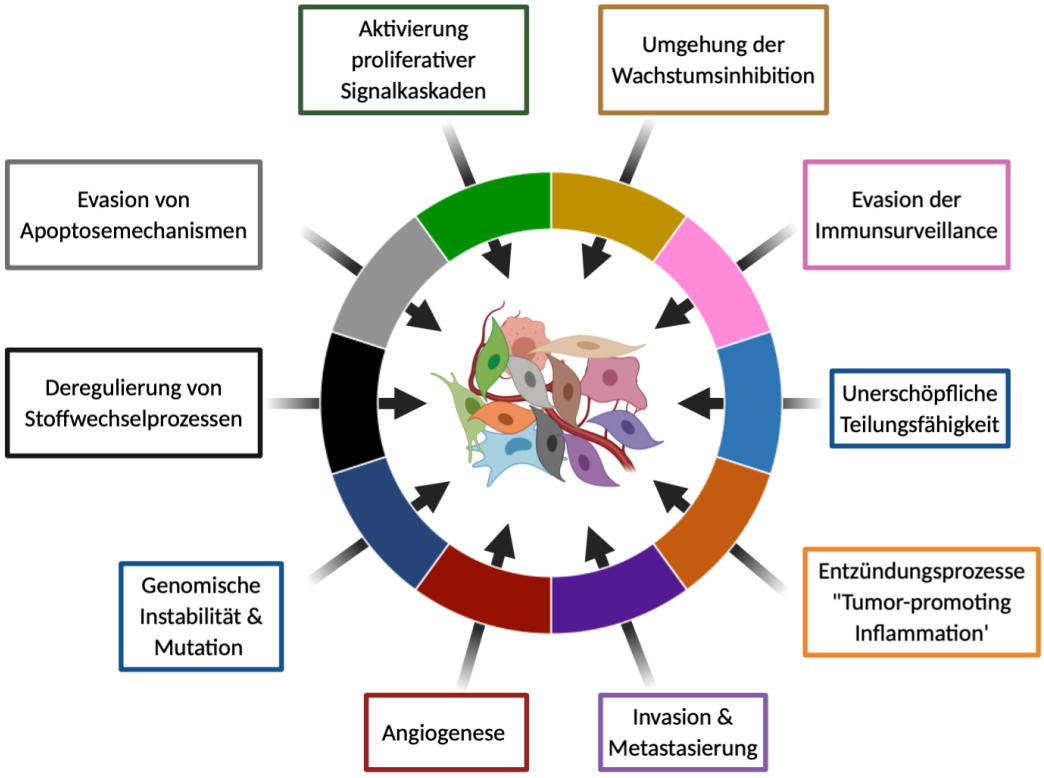

Abbildung 3: ***Hallmarks of cancer: the next generation*** (modifiziert nach Hanahan and Weinberg 2011).

Unterschiedlichste Akteure sind an der Entwicklung dieses strukturierten aber dennoch komplexen Netzwerks der *tumor-promoting inflammation* beteiligt. Reziproke Signalkaskaden zwischen Tumorzellen und Epithel-, Stroma- und Immunzellen führen zu einer kontinuierlichen Rekrutierung von sowohl ausgereiften aber auch unreifen Immunzellen in das Tumorgewebe. Diese Immunzellen können wiederum pro- oder antitumorale Eigenschaften aufweisen. Verschiedene Subgruppen von Entzündungsmediatoren agieren hierbei als zentrale Regulatoren der Entwicklung dieser neoplastischen Inflammationskaskade und tragen weiterhin entscheidend zum Erhalt der Kaskade bei. So nimmt beispielsweise die Chemokin-Superfamilie, als Masterregulator von chemotaktischen Signalen, eine essentielle Rolle bei der Rekrutierung von Immunzellen ein. In folgenden Abschnitten soll detaillierter auf die Eigenschaften und Funktionen zweier weiterer Zytokinfamilien, denen entscheidende Beteiligung am Aufbau der *tumor-promoting inflammation* nachgewiesen werden konnte, eingegangen werden.

4.2 Die Interleukin-1-Familie

1974 beschrieben Dinarello, et al. erstmals zwei unterschiedliche „*human leukocytic pyrogens*“, welche als Schlüsselmediatoren für die Entwicklung von Fieber angesehen wurden (Dinarello, et al. 1974). 1984 und 1985 wurden erstmals die Aminosäure-sequenzen dieser endogenen Pyrogene veröffentlicht, welche seitdem Interleukin-1α und Interleukin-1β genannt wurden (Auron, et al. 1984, March, et al. 1985). In den darauffolgenden Jahrzehnten konnte die essentielle Rolle der Zytokine bei einer Vielzahl von unterschiedlichen Erkrankungen und physiologischen Prozessen nachgewiesen werden. Darüber hinaus wurden einige weitere Mediatoren mit ähnlichen charakteristischen Eigenschaften identifiziert, die heutzutage als Teil der großen IL-1-Familie angesehen werden.

4.2.1 Aufbau und Organisation der IL-1-Familie

Die IL-1-Familie, eine inhomogene Gruppe von Zytokinen mit pleiotropen Funktionen, umfasst aktuell elf Mitglieder. Tabelle 2 zeigt die verschiedenen Zytokine, ihre Funktionen und die zugehörigen Rezeptoren. Sieben dieser elf Moleküle besitzen pro-inflammatorische Wirkungen (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β und IL-36γ), während drei Zytokine (IL-1Ra, IL-36Ra, IL-38) als Rezeptorantagonisten agieren. Eine Sonderstellung besitzt Interleukin-37 (IL-37), welches vor allem auf das angeborene Immunsystem eine ausgeprägte anti-inflammatorische Wirkung ausübt (Garlanda, et al. 2013).

Die hier vorliegende Arbeit konzentriert sich vor allem auf die biologischen Funktionen der Zytokine IL-1α, IL-1β und IL-37.

Tabelle 2: Übersicht der Hauptfunktionen und Rezeptoren der IL-1-Familie (modifiziert nach Garlanda, et al. 2013).

Zytokin	Rezeptor	Ko-Rezeptor	Funktion
IL-1α	IL-1R1 IL-1R2	IL-1RAcP	Alarmierungsfunktion, Inflammation, T_h17 Immunantwort
IL-1β	IL-1R1 IL-1R2	IL-1RAcP	Antimikrobielle Resistenz, Inflammation, T_h17 Immunantwort
IL-18	IL-18Rα	IL-18Rβ	Inflammation, T_h1 Immunantwort
IL-33	ST2	IL-1RAcP	Inflammation, T_h2 Immunantwort
IL-36α	IL-1Rrp2 (IL-36R)	IL-1RAcP	
IL-36β	IL-1Rrp2 (IL-36R)	IL-1RAcP	Entzündungsprozesse der
IL-36γ	IL-1Rrp2 (IL-36R)	IL-1RAcP	Haut und Lunge
IL-1Ra	IL-1F3	IL-1R1	Inhibitor der Entzündungskaskade
IL-36Ra	IL-1Rrp2 (IL-36R)		Inhibitor der Entzündungskaskade
IL-37	IL-18Rα		Inhibitor der Entzündungskaskade
IL-38	IL-1Rrp2 (IL-36R)		Inhibitor der
	TIR8		Entzündungskaskade
	TIGIRR-1		Rolle von IL-1R1 noch
	TIGIRR-2		nicht abschließend geklärt
	IL-1R1 (?)		

4.2.2 Biologie von IL-1α und IL-1β

IL-1α und IL-1β beeinflussen nahezu jede Zelle und viele unterschiedliche Organe des menschlichen Körpers. Durch Wirkung am Hypothalamus tragen die Zytokine beispielsweise zur Entwicklung von Fieber bei (Reimers, et al. 1994, Zheng, et al. 1995). In der Leber initiieren sie die Akute-Phase-Reaktion und sind somit essentieller Bestandteil der angeborenen humoralen Immunantwort (Garlanda, et al. 2013). Um die Effekte von IL-1 im Kontext der Tumorimmunologie besser verstehen zu können, müssen zunächst die Unterschiede und Gemeinsamkeiten der verschiedenen Vertreter der IL-1-Familie genauer betrachtet werden.

4.2.3 Unterschiede und Gemeinsamkeiten von IL-1α und IL-1β

IL-1α und IL-1β werden durch unterschiedliche Gene auf Chromosom 2 kodiert. Sie besitzen durch ihre Bindung an den gleichen Rezeptor ähnliche biologische Eigenschaften (Dinarello 1996). Die funktionellen Unterschiede der Zytokine ergeben

sich durch die unterschiedlichen Expressionsmuster und distinkten Prozessierungs- und Sekretionsmechanismen der Proteine.

Während IL-1α bzw. das IL-1α-Vorläuferpeptid (pro-IL-1α) vor allem in Epithelzellen konstitutiv exprimiert wird, erfolgt die Bildung von IL-1β weitestgehend durch myeloide hämatopoetische Zellen wie Monozyten, Makrophagen und dendritische Zellen (Mantovani, et al. 2019). Ein weiteres wichtiges Unterscheidungsmerkmal ist die biologische Aktivität der Vorläufermoleküle. Pro-IL-1α ist aktiv und wird im Rahmen von nekrotisierenden Prozessen verstärkt freigesetzt. Es fungiert hierbei als einer der zentralen Mediatoren der Entzündungskaskade und trägt wesentlich zur Ausbildung von sterilen Entzündungen bei (Rider, et al. 2011). Ähnlich wie viele andere Zytokine der IL-1-Familie besitzt auch das 31 Kilodalton (kDa) schwere pro-IL-1α N-terminale Schnittstellen für Restriktionsenzyme (z.B. Granzym B, Neutrophilen Elastase und Calpain), die zu einer Prozessierung von pro-IL-1α führen. *Mature* IL-1α besitzt eine deutlich höhere Rezeptoraffinität, was sich in einer gesteigerten Bioaktivität äußert (Afonina, et al. 2011). Die Prozessierung von IL-1α scheint deshalb ähnlich wie für IL-1β eine wichtige Regulationsfunktion einzunehmen (Afonina, et al. 2015).

Neben einer extrazellulären Wirkung ist für pro-IL-1α auch eine nukleäre Funktion beschrieben. Nach Translokation in den Nukleus kann pro-IL-1α die Induktion und Aktivierung der zentralen pro-inflammatorischen Transkriptionsfaktoren *nuclear factor kappa B* (NF-kB) und *activator protein 1* (AP-1) fördern (Hu, et al. 2003, Werman, et al. 2004). Darüber hinaus können durch Interaktion von transmembran-verankertem IL-1α auf Makrophagen und Muskelzellen pro-inflammatorische Signalkaskaden ausgelöst werden (Kurt-Jones, et al. 1985, Sasu, et al. 2001).

Im Gegensatz dazu besitzt das 31 kDa schwere Interleukin-1β-Vorläuferpeptid (pro-IL-1β) noch keine biologische Wirksamkeit. Es unterliegt vielmehr einem außergewöhnlichen mehrstufigen Aktivierungsprozess: Im ersten Schritt kommt es durch die Aktivierung von z. B. *Toll-like*-Rezeptoren (TLR) zu einer transkriptionellen Hochregulation von pro-IL-1β sowie zu einer verstärkten Bildung eines zytosolischen Multiproteinkomplexes, dem Inflammasom. Durch ein zweites noch nicht gänzlich entschlüsseltes Signal kommt es zu einer Aktivierung des Inflammasoms und zu einer Caspase-1-vermittelten proteolytischen Prozessierung von pro-IL-1β zu seiner aktiven maturen Form (vgl. Abbildung 4, Gaidt, et al. 2016). Neben diesem klassischen Weg wurden in den letzten Jahren außerdem nicht-klassische (*non-canonical*) Prozessierungswege beschrieben, auf die hier nicht näher eingegangen wird (Afonina,

et al. 2015). *Mature* IL-1β wird anschließend über Golgi-Apparat-unabhängige Mechanismen aus der Zelle ausgeschleust (Lopez-Castejon and Brough 2011). IL-1α besitzt somit am ehesten die Rolle eines lokalen Mediators von Entzündungen, während IL-1β systemische Entzündungsreaktionen moduliert.

4.2.4 IL-37 als Inhibitor der angeborenen Immunität

Unter den Zytokinen der IL-1-Familie nimmt IL-37 eine Sonderstellung ein. Es besitzt eine ausgeprägte immunsuppressive Wirkung, v.a. auf das angeborene Immunsystem. So konnten Nold, et al. nach *small interfering RNA*-vermittelter Inhibition der IL-37 Funktion in primären *peripheral blood mononuclear cells (*PBMC) eine signifikante Erhöhung von sezernierten pro-inflammatorischen Zytokinen (u.a. IL-1α, IL-6) demonstrieren. Überexpression von IL-37 in verschiedenen monozytären (THP-1, *RAW Macrophages*) bzw. epithelialen Zelllinien (A549) führte im gleichen Sinne zu einer deutlichen Minderung der spontanen oder Lipopolysaccharid (LPS)-induzierten Sekretion inflammatorischer Mediatoren. Darüber hinaus zeigten transgene IL-37 exprimierende Mäuse – ein murines IL-37 Homolog konnte bislang noch nicht identifiziert werden – ausgeprägte Resistenz gegenüber applizierten Stimuli in unterschiedlichen inflammatorischen Mausmodellen (Nold, et al. 2010). Es existieren fünf unterschiedliche Splicevarianten von IL-37: IL-37a-e. IL-37-Expression konnte mittels immunhistochemischer Methoden in gesunden Immunzellen (Monozyten, Makrophagen, Plasmazellen) und Epithelzellen (Haut, Niere, Darm) sowie in neoplastischen Zellen nachgewiesen werden. Monozyten exprimieren IL-37 nicht konstitutiv, sondern vor allem nach Stimulation mit IL-1β, TLR Agonisten oder TGF-β (Dinarello, et al. 2016).

IL-37 bindet sowohl die α-Kette des IL-18-Rezeptors (IL-18Rα) als auch das IL-18-bindende Protein (IL-18BP). Bufler, et al. konnten demonstrieren, dass IL-37 zu einer Verstärkung der inhibitorischen Effekte von IL-18BP führt (Bufler, et al. 2002). Für die Vermittlung der anti-inflammatorischen Eigenschaften von IL-37 scheint allerdings die Interaktion von IL-37 mit IL-18Rα eine zentrale Rolle zu spielen. Nach Bindung von IL-37 wird der *orphan decoy* Rezeptor IL-R8 rekrutiert, was zu einer Hemmung der pro-inflammatorischen, *myeloid differentiation primary response 88* (MYD88)-vermittelten Signalkaskade führt (Li, et al. 2015, Nold-Petry, et al. 2015). Pro-IL-37 wird wie IL-1β, IL-18 und IL-33 durch Caspase-1 prozessiert. *Mature* IL-37 wird nach N-terminaler Caspase-vermittelter Prozessierung zwischen den Aminosäuren D20 (Asparaginsäure) und E21 (Glutaminsäure) aus pro-IL-37 gebildet. Prozessiertes IL-37 wird aktiv in den Nukleus transportiert (Bulau, et al. 2014), weshalb eine Wirkung auf transkriptioneller

Ebene für IL-37 vermutet wird (Sharma, et al. 2008). Die genauen regulatorischen Mechanismen sind bislang allerdings noch nicht geklärt. Auch die genauen Anteile dieser nukleären Funktion auf die inhibitorischen Effekte von IL-37 lassen sich schwer abschätzen. IL-37 wird wie die meisten anderen Zytokine der IL-1-Familie auch durch Golgi-Apparat-unabhängige Mechanismen sezerniert. Die genauen Mechanismen sind allerdings auch hier noch nicht vollständig entschlüsselt. Nach LPS Stimulation von Monozyten konnte vor allem pro-IL-37 im Überstand nachgewiesen werden, während nach Stimulation mit ATP und der daraus resultierenden Inflammasom und Caspase-1-Aktivierung (Signal 2, vgl. Abbildung 4**)** auch prozessiertes IL-37 nachweisbar war (Bulau, et al. 2014).

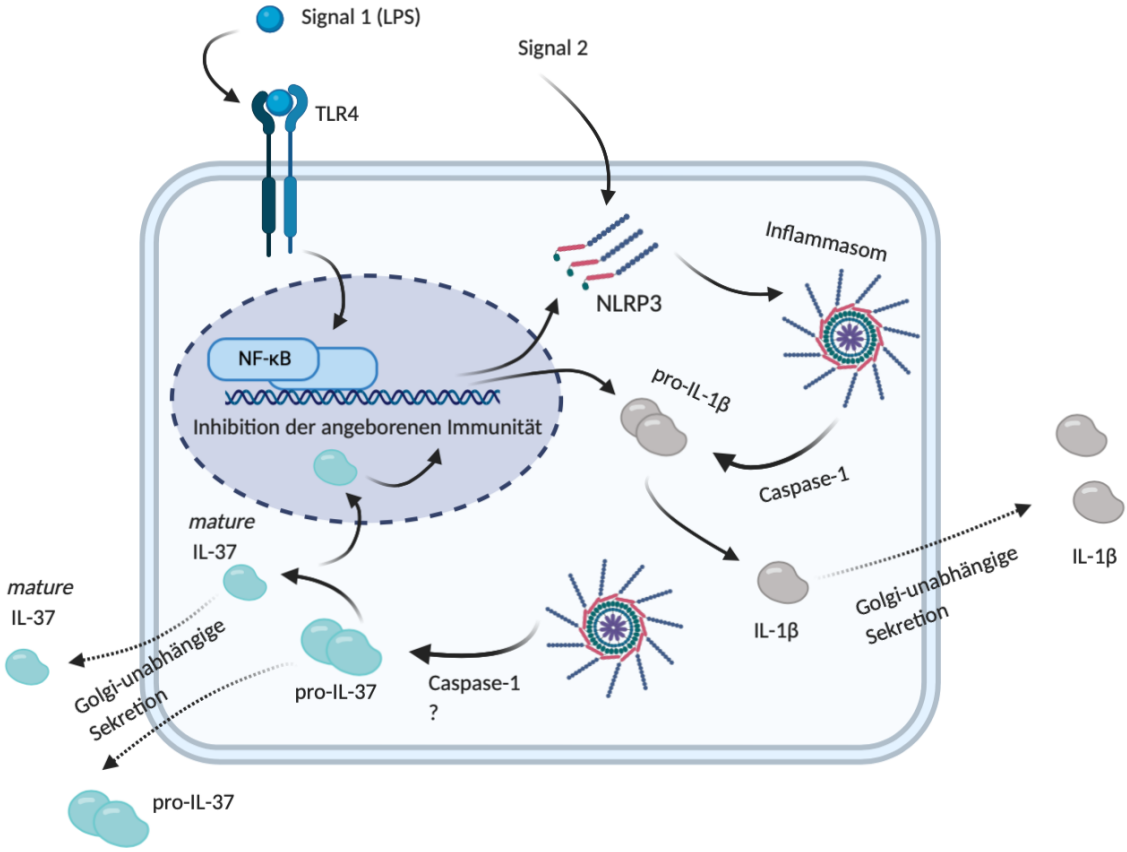

Abbildung 4: Prozessierung, Sekretion und nukleäre Translokation von IL-1β und IL-37 (modifiziert nach Afonina, et al. 2015).

4.2.5 Die IL-1-Zytokinfamilie im Kontext der Tumorbiologie

Die Zytokine der IL-1-Familie scheinen in nahezu jeder Entwicklungsstufe von malignen Erkrankungen wichtige Funktionen zu erfüllen. So konnte in verschiedenen klinischen Studien die gesteigerte Expression der Zytokine mit einer Prognose-verschlechterung des Gesamtüberlebens korreliert werden (Barber, et al. 2000, Huo, et al. 2017, Zhang, et al. 2019).

Auch bei der Initiierung von Neoplasien im Rahmen von chronischen Entzündungsprozessen wurde der IL-1-Familie zentrale Funktionen zugeschrieben. So trägt beispielsweise der IL-1β-IL-1R-Signalweg zur Entwicklung von Asbestose-induzierten Mesotheliomen bei (Kadariya, et al. 2016). Auch *single nucleotid polymorphisms* im IL-1β-Gen wurden mit einem erhöhten Risiko für die Entwicklung von *non-small cell lung cancer* assoziiert (Zienolddiny, et al. 2004). Für IL-37 konnte in murinen Kolitis-induzierten Karzinommodellen durch transgene Expression von IL-37 in Mäusen eine Inhibition der Formation von Adenomen und Karzinomen gezeigt werden (Mountford, et al. 2019).

Zentrale Auswirkungen besitzen die Signalmoleküle auch auf das Fortschreiten von Neoplasien. Das durch Tumor- und Stromazellen freigesetzte IL-1 fördert die Vaskularisation des Tumorgewebes durch Induktion pro-angiogener Faktoren wie *vascular endothelial growth factor* (VEGF) und *fibroblast growth factor* (FGF) (Carmi, et al. 2009, Voronov, et al. 2003). Ge, et al. legten dar, dass die Überexpression von IL-37 in humanen Lungenkarzinomzellen (H1299 Zellen) zu einer reduzierten Tumorvaskularisierung *in vivo* führt. *In vitro* konnten darüber hinaus direkte hemmende Effekte von IL-37 auf das Wachstum und die Formation von Kapillaren von *human umbilical vein endothelial cells* (HUVEC) Zellen demonstriert werden (Ge, et al. 2016).

Auto- und juxtakrine Sekretion von IL-1 führt auf malignen Zellen durch Aktivierung pro-inflammatorischer Kaskaden zur Tumorzellinvasion, Migration und Proliferation (Ling, et al. 2012). Für IL-37 wurden im Gegensatz dazu u.a. hemmende Effekte auf den *wingless and INT-1/ β-Catenin* (WNT/β-Catenin)- und den *signal transducers and activators of transcription* drei (STAT3)-Signalweg beschrieben (Wang, et al. 2015, Wu, et al. 2018, Yan, et al. 2017).

Wichtige Rollen spielen IL-1α und IL-1β – sowohl direkt als auch indirekt – auch bei der Etablierung und Erhaltung des immunosuppressiven Tumormikromilieus. Schon in frühen Studien wurde die Involvierung von IL-1 in die Hämatopoese sowie die Rekrutierung von Immunzellen aus dem Knochenmark demonstriert (Dinarello 1996). Im tumorbiologischem Kontext fördern IL-1α und IL-1β die Rekrutierung von *myeloid-derived suppressor cells* (MDSC) (Elkabets, et al. 2010, Mantovani 2010). Weiterhin konnte in Studien gezeigt werden, dass die IL-1-Signalkaskade wichtige Funktionen beim Erhalt der suppressiven Eigenschaften von Tumor-assoziierten Makrophagen (TAM) erfüllt (Mantovani, et al. 2018, Pan, et al. 2017). In regulatorischen T-Zellen (Treg) von malignen Melanompatienten (MM) wurde eine

hohe IL-37 Expression nachgewiesen, wobei die genaue Funktion in diesem Kontext noch nicht vollständig geklärt ist (Osborne, et al. 2019).

Auch auf die unterschiedlichen Stufen der Metastasierungskaskade scheinen die Zytokine der IL-1-Familie Einfluss zu nehmen. So konnten Giavazzi, et al. in humanen MM Xenograft Modellen eine signifikante Verstärkung der Lungenmetastasierung nach IL-1 Gabe zeigen (Giavazzi, et al. 1990), während Holen, et al. im gleichen Sinne eine Reduktion der Metastasierung von humanen Brustkarzinomzellen durch Therapie mit dem IL-1Ra Anakinra demonstrierten (Holen, et al. 2016). Im Gegensatz dazu führte die Überexpression von IL-37 in humanen Lungenkarzinomzellen (A549) durch Inhibition des *ras-related C3 botulinum toxin substrate* 1 Proteins zu einer reduzierten Anzahl an Lungenmetastasen (Li, et al. 2018).

Abbildung 5 soll eine Übersicht über die bisher beschriebenen Wirkungen von IL-1β und IL-37 auf die verschiedenen Stadien der Tumorentwicklung bieten.

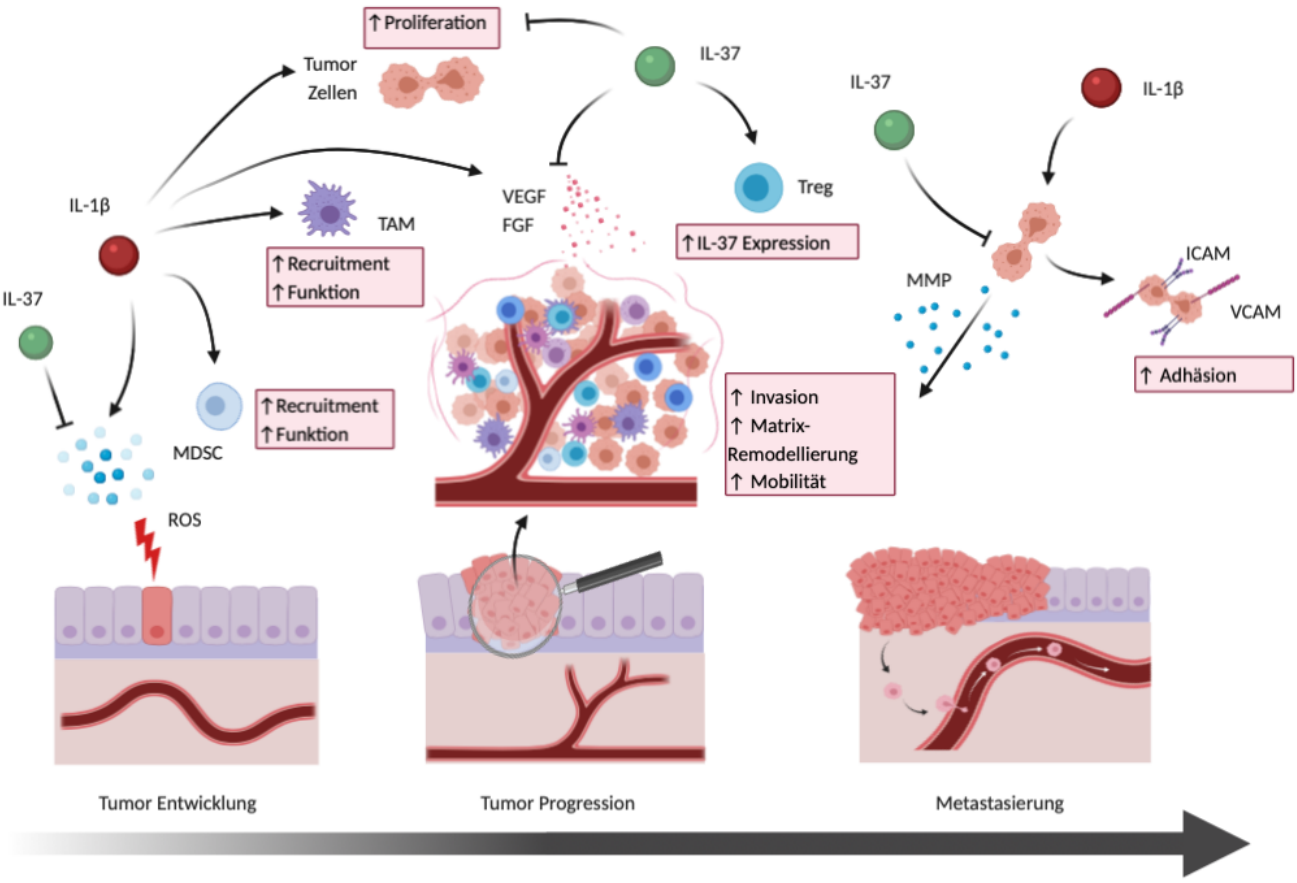

Abbildung 5: Übersicht über die Wirkungen von IL-1 und IL-37 auf verschiedene Stadien der Tumorentwicklung (in Anlehnung an Gottschlich, et al. 2018).

Neben den hier skizzierten pro- bzw. anti-neoplastischen Wirkungen der IL-1-Zytokine konnten wir in unserer Veröffentlichung (Voigt, et al. 2017) eine weitere wichtige Funktion von IL-1α und IL-1β – die Regulation der IL-22-Sekretion – aufzeigen. Um das komplexe Zusammenspiel dieser beiden Zytokinfamilien besser verstehen zu können, sollen im Folgenden die Funktionen von IL-22 als Mitglied der IL-10-Familie näher erläutert werden.

4.3 IL-22: Ein Zytokin der IL-10-Familie

Die Zytokine der IL-10-Familie leisten entscheidenden Beitrag zur Aufrechterhaltung von unterschiedlichen fundamentalen regulatorischen und homöostatischen Prozessen.

4.3.1 Aufbau und Organisation der Interleukin-10-Familie

Die IL-10-Familie umfasst neun Mitglieder: IL-10, IL-19, IL-20, IL-22, IL-24, IL-26 und die entfernteren Zytokine IL-28A, IL-28B und IL-29, welche auch als Typ-III-Interferone bezeichnet werden (Ouyang and O'Garra 2019). Die durch die Moleküle ausgelösten Signalkaskaden spielen eine wichtige Rolle im Rahmen von Infektionen und Entzündungsreaktionen, bei der Homöostase und Regeneration verschiedener Gewebe und im Rahmen von Autoimmunprozessen. Darüber hinaus konnte in den letzten Jahren auch eine wichtige Rolle der Zytokine bei der Entwicklung und dem Fortschreiten von Tumorerkrankungen festgestellt werden.

4.3.2 Die IL-22-IL-22R1-Achse

IL-22 wird ausschließlich durch Immunzellen unterschiedlicher Art gebildet. Die CD4+ Typ17-T-Helferzellen (T_h17) und T_h22 Subgruppen sind die Hauptproduzenten von IL-22, aber auch CD8+ Typ17 zytotoxische T-Zellen (T_c17), gamma delta T-Zellen (γδ T-Zellen), Natürliche-Killer-T-Zellen (NKT Zellen) und *Innate lymphoid cells* (ILC) tragen zur Bildung von IL-22 beim Menschen bei (Lim and Savan 2014, Ouyang and O'Garra 2019). Tabelle 3 gibt eine Übersicht über die unterschiedlichen Immunzell-subtypen, denen eine Produktion von IL-22 nachgewiesen werden konnte.

Tabelle 3: Übersicht der wichtigsten IL-22 produzierenden Zelltypen (modifiziert nach Markota, et al. 2018).

Zelle	Oberflächen-marker	Weitere Zytokine	Regulatoren	Transkriptionsfaktoren
T_h1	CD3+ CD4+	IFNγ, IL-2, TNFα, β	IL-12, IL-18 (IL-12, IL-23)	STAT4
T_h17	CD3+ CD4+	IL-17 A/F. IL-21, IL-26	IL-1β, IL-6, IL-21, IL-23, TGFβ	STAT3, RORγt, Notch, AhR
T_h22	CD3+ CD4+	-	IL-6, TNFα (IL-6, IL-21)	STAT3, AhR
T_c17	CD3+ CD8+	IFNγ, IL-17	unkown (IL-23)	STAT3
T_c22	CD3+ CD8+	-	IL-21 unkown,	STAT1,3,5
γδ T Zellen	CD3+, γ,δ-TCR+	IL-17, IL-21	IL-23 (IL-23, IL-1β)	STAT3, RORγt, AhR
NKT	CD3-	IFNγ, IL-17	IL-23, IL-1β	STAT3, RORγt, AhR

Die Signalkaskade wird durch Bindung von IL-22 an seinen heterodimeren Rezeptorkomplex bestehend aus dem IL-22R1 und dem IL-10 Rezeptor 2 (IL-10R2) ausgelöst (Kotenko, et al. 2001). Nach Bindung an den entsprechenden Rezeptorkomplex kommt es zur Aktivierung von STAT1, STAT3, STAT5, NF-kB, *mitogen-activated protein kinase* (MAPK) und *phosphatidylinositide 3-kinase-Akt-mammalian target of rapamycin* (PI3K-Akt-mTOR) (Sabat, et al. 2014).

Die engmaschige Regulation und Feinadjustierung der IL-22 Funktion wird zusätzlich durch Sekretion des natürlichen Inhibitors IL-22-bindendes Protein (IL-22BP, IL-22RA2) gesteuert. IL-22BP ist ein einzelkettiges Protein, bestehend aus 210 Aminosäuren. Durch die hochaffine Bindung von IL-22BP an sezerniertes ungebundenes IL-22 (20-1000 Fach höhere Affinität als IL-22R1) kann es die Bindung von IL-22 an den Transmembranrezeptor verhindern und fungiert so als zentraler Inhibitor der IL-22 Funktion (Sabat, et al. 2014).

Obwohl IL-22 fast überwiegend durch Immunzellen sezerniert wird (vgl. Tabelle 3), wird IL22R1 ausschließlich auf Nicht-Immunzellen exprimiert und entfaltet dort auch seine Wirkung. Vor allem auf Hepatozyten, Keratinozyten, sowie mukosalen und pankreatischen Epithelzellen konnte eine signifikante IL-22R1 Expression nachgewiesen werden (Aggarwal, et al. 2001, Boniface, et al. 2005, Brand, et al. 2007, Feng, et al. 2012).

4.3.3 Physiologische Funktionen von IL-22

Physiologisch setzt IL-22 Signalkaskaden in Gang, die das Eindringen und die Invasion von Pathogenen verhindern sollen. So konnten Aujila, et al. die fundamentale Rolle von Th17-Zellen und ihren Effektorzytokinen IL-17 und IL-22 bei bakteriellen, gram-negativen Pneumonien nachweisen (Aujla, et al. 2008). Nur IL-22, nicht IL-17, führte zusätzlich zu einer verstärkten Formation klonaler Kolonien und zur verstärkten transepithelialen Wundheilung in primären humanen Bronchialzellen (*primary human bronchial epithelial cells*, HBE Zellen). Ähnliche proliferative und migratorische Effekte konnten auch für andere mukosale bzw. epitheliale Zelltypen beschrieben werden, u.a. Keratinozyten, Hepatozyten und Kolonepithelzellen (Boniface, et al. 2005, Brand, et al. 2007, Pickert, et al. 2009). IL-22 fungiert somit als zentraler Mediator von proliferativen Signalwegen und trägt essentiell zur Instandhaltung einer intakten epithelialen Barriere – einem Grundpfeiler der angeborenen Immunität – bei.

Weiterhin induziert IL-22 in Hepatozyten die Ausschüttung von Akute-Phase-Proteinen, wie z.B. Serum Amyloid A (SAA), Fibrinogen und Haptoglobin (Liang, et al. 2010). IL-22 fördert die Bildung der Chemokine CXCL1, CXCL2, CXCL5 und CXCL8 und kann so die Rekrutierung von Neutrophilen Granulozyten induzieren (Wolk, et al. 2009). Diese erfüllen wiederum essentielle Aufgaben in der Abwehr von Pathogenen. Darüber hinaus fördert IL-22 die Sekretion anti-mikrobieller Substanzen, wie z.B. der S100A Proteine (S100A7-9), Defensine (β-Defensin 2, BD2) und Lipocalin 2 (LCN2) (Wolk, et al. 2006).

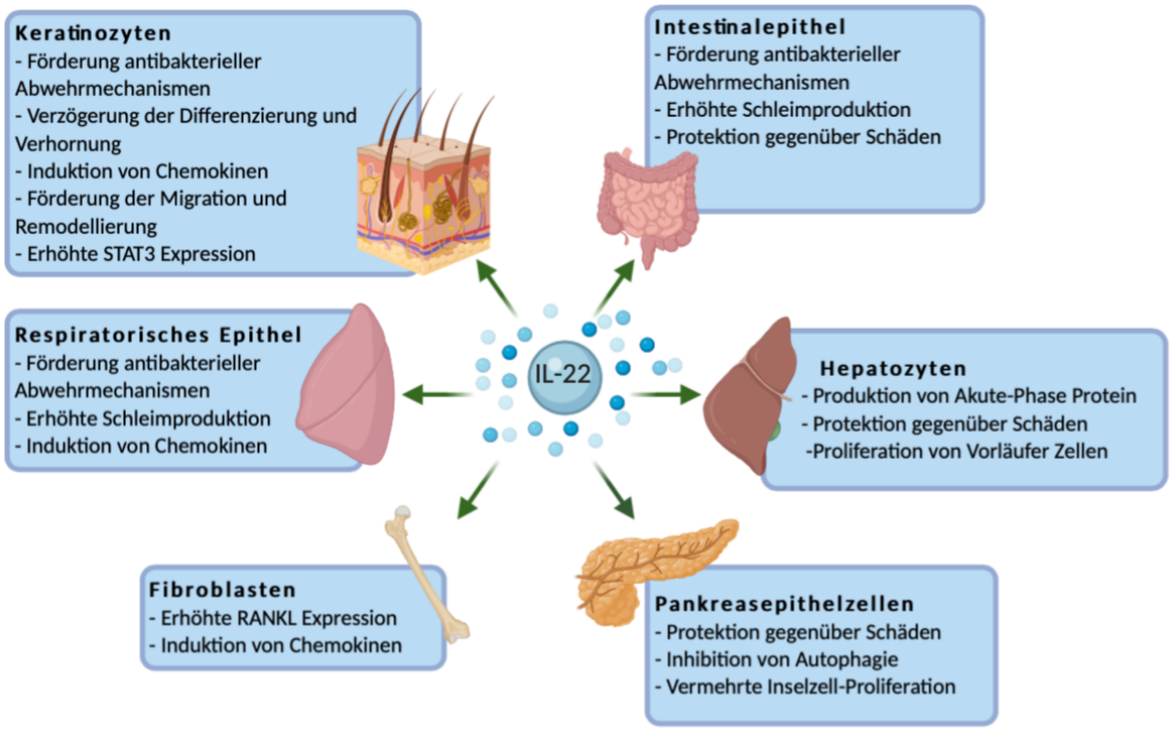

Abbildung 6: Physiologische Funktionen von IL-22 (modifiziert nach Sabat, et al. 2014).

4.3.4 Die IL-22-IL-22R1-Achse im Kontext der Tumorbiologie

Die nahezu explizite Wirkung von IL-22 auf Zellen epithelialen Ursprungs und die zentralen regulatorischen Funktionen von IL-22 auf Proliferation, Migration und Bewegung gesunder Zellen verdeutlichen das Potenzial für maligne Zellen, die Signalkaskaden auszubeuten. Eine Vielzahl von Publikationen demonstrieren die Expression des heterodimeren IL-22R1-IL-10R2-Komplex auf Tumorzellen und eine durch IL-22 induzierbare Förderung von Proliferation, Migration und Invasion maligner Zellen (Curd, et al. 2012, Kobold, et al. 2013, Zhang, et al. 2008).

Bi, et al. stellten beispielsweise dar, dass IL-22 die Proliferation und Migration von Lungenkarzinomzellen fördert (Bi, et al. 2016), während Jiang, et al. die signifikante Beteiligung der IL-22-IL-22R1-STAT3-Achse und ihrer *downstream* Effektormoleküle auf die Entwicklung, Progression und Metastasierung des Hepatozellulären Karzinoms nachweisen konnten. Vorarbeiten unserer Arbeitsgruppe illustrierten außerdem, dass IL-22 in der Lage ist, das Wachstum von Chemotherapie-resistenten Lungenkarzinom-zellen zu fördern (Kobold, et al. 2013). Weitere Publikationen beschreiben die Involvierung der IL-22 Signalkaskade in die Entwicklung bzw. Progression von Brust- (Kim, et al. 2014), Schilddrüsen- (Mei, et al. 2016) und Kolonkarzinomen

(Nagalakshmi, et al. 2004) sowie nicht-melanotischem Hautkrebs (Nardinocchi, et al. 2015). Darüber hinaus wurden hohe Expressionslevel von IL-22 und IL-22R1 in einigen Studien mit einem signifikant schlechteren Langzeitüberleben assoziiert (Guillon, et al. 2016, Perusina Lanfranca, et al. 2020, Waidmann, et al. 2014, Wen, et al. 2014, Qin, et al. 2014). Hohe IL-22-Spiegel im Serum wurden außerdem mit einer erhöhten Chemotherapie-Resistenz bei Kolonkarzinompatienten korreliert (Wu, et al. 2013).

Huber, et al. waren in der Lage die zentrale Rolle von IL-22BP in der Tumorigenese von Kolonkarzinomen zu illustrieren. *NLR family pyrin domain containing 3 bzw. 6* (NLRP3, 6) getriggerte Freisetzung von IL-18 führte zu einer Herunterregulation von IL-22BP *in vivo*. Die exzessive Aktivierung der IL-22-induzierten Signalkaskaden führte zu einer überschüssigen epithelialen Proliferation und zu einer verstärkten Bildung von malignen Neoplasien des Kolons (Huber, et al. 2012). Abbildung 7 soll einen Überblick über die nachgewiesenen Effekte von IL-22 auf verschiedene Tumorentitäten geben.

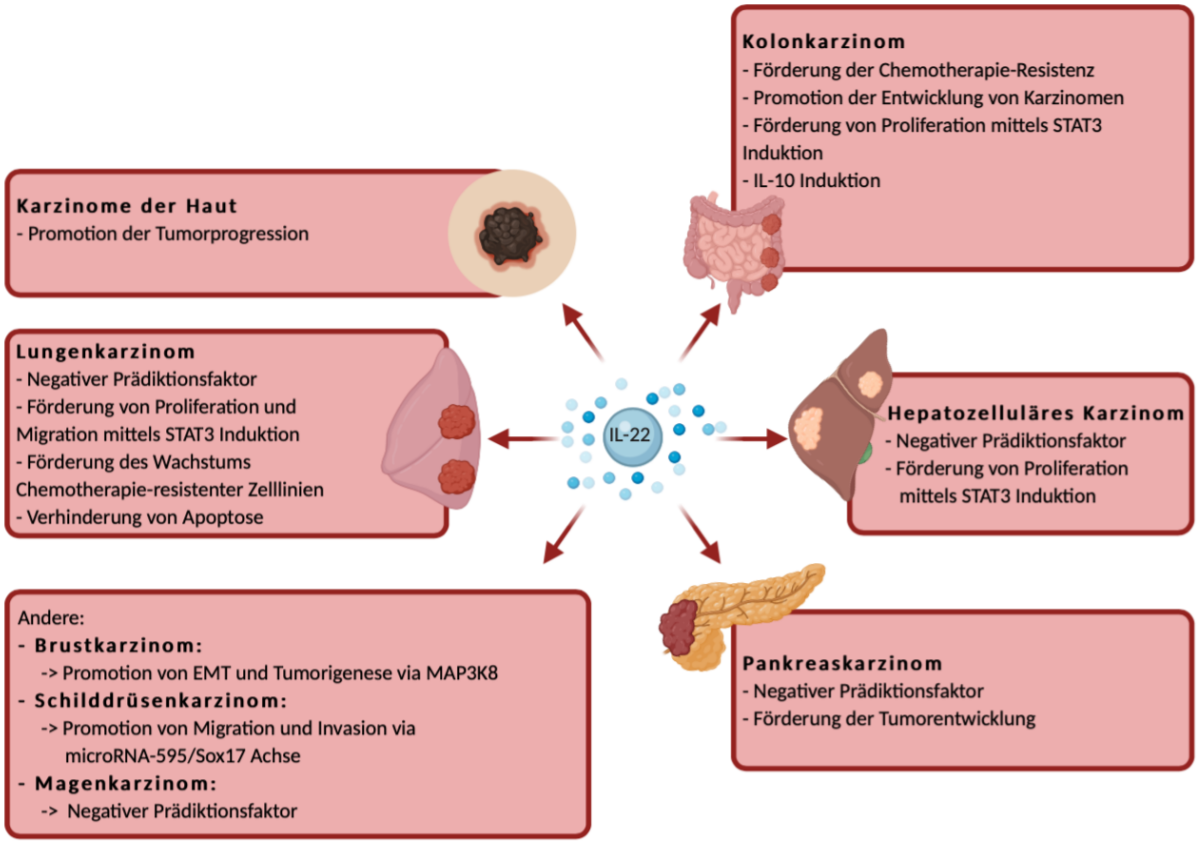

Abbildung 7: Nachgewiesene Effekte von IL-22 auf verschiedene Tumorentitäten (in Anlehnung an Sabat, et al. 2014).

4.4 Fragestellung der Arbeit

Sowohl pro- als auch anti-inflammatorische Zytokine fungieren als fundamentale Mediatoren zwischen den unterschiedlichen Zellen des Tumormikromilieus. Das genaue Verständnis der zu Grunde liegenden Regulations- und Wirkmechanismen ist essentiell, um sich die gezielte Modulation dieser Prozesse für die Therapie von Neoplasien zu Nutze zu machen.

Die IL-1-Zytokinfamilie erfüllt durch ihre Heterogenität und ihre Pleiotropie zentrale Funktionen in der Regulation des Tumormikromilieus und ist deshalb in den letzten Jahren immer stärker in den Fokus der Immuntherapie gerückt (Gottschlich, et al. 2018). Essentiell für die Entwicklung neuer Therapeutika und die Anwendung schon etablierter anti- bzw. pro-inflammatorischer Medikamente in der Tumortherapie ist jedoch ein detailliertes Verständnis der Regulationsprozesse und der Wirkweisen der zu beeinflussenden Mediatoren.

Aus diesem Grund befasst sich folgende Arbeit sowohl mit (1) dem komplexen Zusammenspiel von Tumorzellen und Zytokinen der IL-1 und der IL-10-Familie, wie auch (2) einer detaillierten Untersuchung der biophysikalischen, biochemischen und biologischen Grundlagen von IL-37, als Mitglied der IL-1-Familie und zentraler Mediator anti-inflammatorischer Immunantworten.

5. Zusammenfassung

Die vorliegende kumulative Dissertation fast die Ergebnisse zweier publizierter Originalarbeiten zusammen. Beide Arbeiten untersuchen die Rolle pro- und anti-inflammatorischer Zytokine im tumorbiologischen Kontext, ihre regulatorischen Funktionen im Tumormikromilieu sowie distinkte zytokinspezifische Wirkungsmechanismen.

Die erste Publikation untersucht die Regulation der Produktion des Zytokins IL-22 in Brust- und Lungenkarzinom-Modellen. Hierbei konnte ich zunächst in murinen Modellen die IL-1α- und IL-23-abhängige IL-22-Induktion aus Splenozyten demonstrieren. In humanen Modellen war ich in der Lage das vor allem durch myeloide Immunzellen produzierte IL-1β als Schlüsselzytokin der Regulation der IL-22-Produktion zu identifizieren (Abbildung 8). Darüber hinaus konnte ich die Beteiligung des NLRP3-Inflammasoms in der Regulation von IL-22 zeigen. Die *in vitro* generierten Befunde an murinen und humanen Tumorzelllinien ergänzte ich durch die Untersuchung von primären humanen Lungenkarzinomproben (Voigt, et al. 2017).

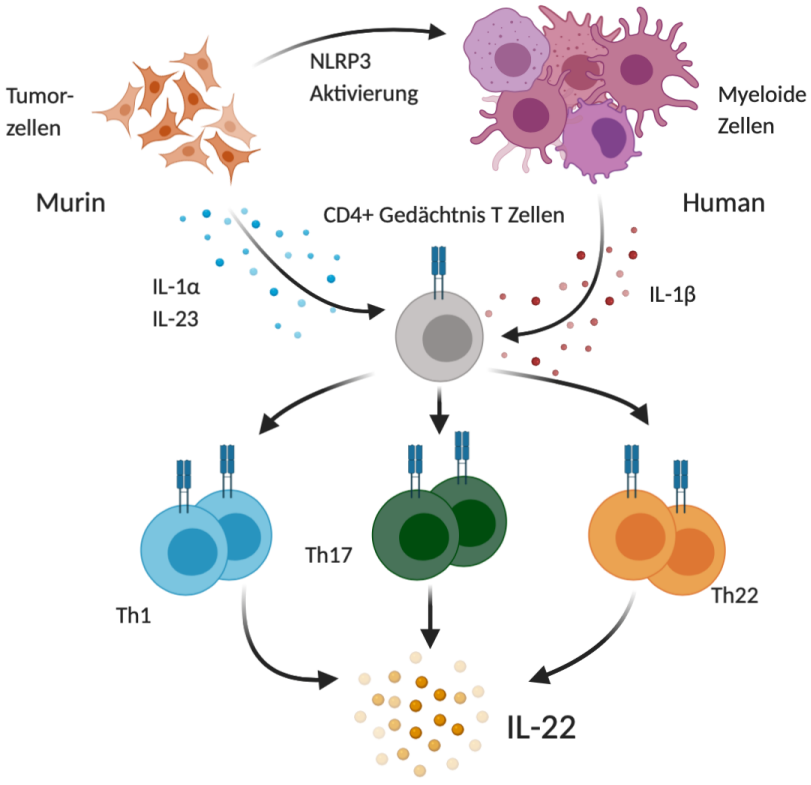

Abbildung 8: Regulation der IL-22 Produktion in Brust- und Lungenkarzinomen (modifiziert nach Voigt, et al. 2017).

Die zweite Publikation untersucht die Rolle von IL-37 als Inhibitor der angeborenen Immunität. Durch die Kombination von biophysikalischen, biochemischen und biologischen Methoden und Modellen gelang es uns, distinkte Funktionen von IL-37 näher zu beschreiben. Hierbei konnten wir in *in vitro* Experimenten zeigen, dass durch gezielten Austausch einzelner Aminosäuren im IL-37-Polypeptid die Dimerisierung des IL-37-Proteins verhindert und somit eine Wirkverstärkung gegenüber der nativen Form des Proteins im nanomolaren Bereich erzielt werden kann (Eisenmesser, et al. 2018; vgl. Abbildung 9).

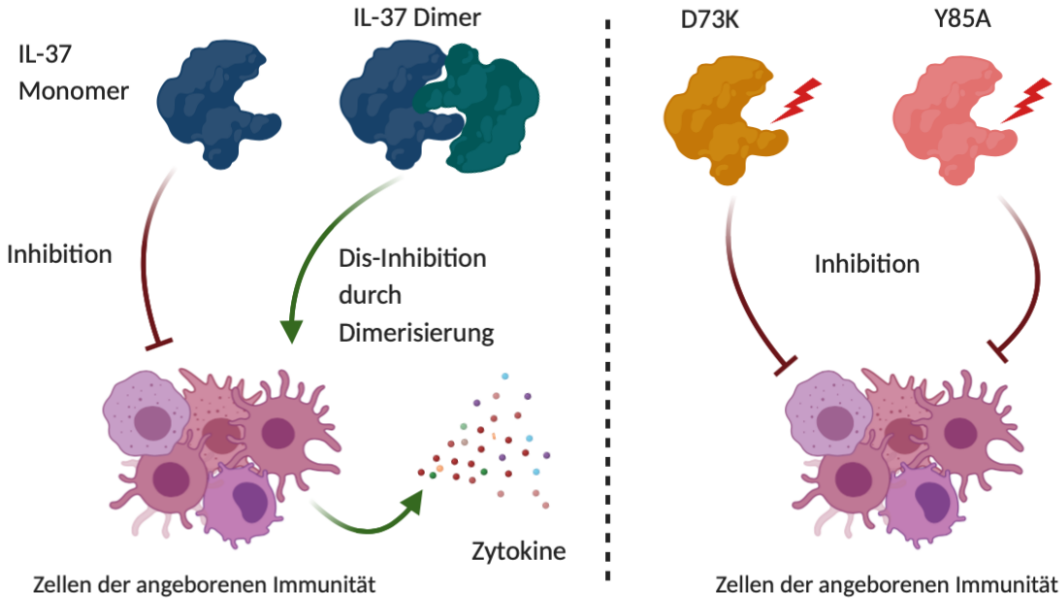

Abbildung 9: Wirkungsweise von rekombinantem IL-37 und Mutanten.

Beide Arbeiten sollen das Verständnis der Zytokinbiologie fördern und können einen Beitrag zur Entwicklung neuer Therapeutika in onkologischen und nicht-onkologischen Erkrankungen leisten. Zum einen konnte ich im Rahmen der ersten Publikation eine bisher wenig beachtete Wirkung von IL-1α und IL-1β – die Förderung der IL-22 Produktion – identifizieren und so die Vorteile der therapeutischen Beeinflussung der IL-1-IL-1R-Achse in Patienten mit Brust- oder Lungenkarzinomen nochmals hervorheben. Die aus der zweiten Publikationen gewonnenen Erkenntnisse – die Identifikation und Modulation des Protein-Dimer-Interfaces und die daraus resultierende Optimierung des Dosis-Wirkungsprofils von IL-37 – können den Weg für eine optimierte Anwendung von IL-37 zur Inhibierung der angeborenen Immunität ebnen.

6. Abstract

The following cumulative dissertation summarizes the results of two published scientific articles. Both manuscripts scrutinized the fundamental role of pro- and anti-inflammatory cytokines in tumor biology, their role as master regulators of the tumor microenvironment, as well as their characteristic and distinctive mode of actions.

The first publication focuses on the underlying regulatory mechanisms of IL-22 production in models of breast- and lung carcinoma. In the murine setting I could demonstrate the central role of the cytokines IL-1α and IL-23 for the production of IL-22. Intriguingly, the underlying regulatory mechanisms differed in men. This could be shown in human co-culture experiments. Here I could identify myeloid cell-derived IL-1β as the key regulatory cytokine and illustrate the pivotal function of the NLRP3 inflammasome for the production of IL-22 (Figure 8). Finally, I was able to validate the insights generated in murine and human *in vitro* models using primary lung carcinoma tissue samples and healthy control tissue (Voigt, et al. 2017).

The second publication aims to elucidate the role of IL-37 as a fundamental inhibitor of innate immunity. In a combinatory approach utilizing biophysical, biochemical as well as biological methods, we were able to decipher the distinct mode of action of IL-37. I could demonstrate *in vitro* that single amino acid modulations of the IL-37 polypeptide prevent the dimerization of the IL-37 protein, ultimately leading to an enhanced treatment efficacy in nanomolar ranges (Figure 9). Thus, I was able to validate the generated biophysical and biochemical insights using functional assays (Eisenmesser, et al. 2018).

Both publications are supposed to contribute to a more detailed understanding of cytokine biology and contribute to the development of new therapeutic approaches in oncological and non-oncological diseases. In the first publication I was able to shed light on a so far barely described function of IL-1α and IL-1β – orchestrating the production and secretion of IL-22 – thus highlighting the possibilities of therapeutic modulation of the IL-1-IL-1R-axis in patients suffering from breast and lung carcinoma. Moreover, the insights generated in the second publication – the identification and modulation of the protein-dimer interface and the resulting enhancement of the therapeutic efficacy of IL-37 – can potentially pave the way for utilizing the function of the IL-37 protein to therapeutically modulate innate immunity.

7. Publikationen

7.1 Cancer cells induce interleukin-22 production from memory CD4+ T cells via interleukin-1 to promote tumor growth

Im Rahmen der Publikation „*Cancer cells induce interleukin-22 production from memory CD4+ T cells via interleukin-1 to promote tumor growth*" konnten wir die beiden IL-1-Zytokine als zentrale Regulatoren der IL-22 Produktion in murinen und humanen Modellen identifizieren. Darüber hinaus waren wir in der Lage, eine detaillierte immunologische Charakterisierung der Hauptproduzenten von IL-22 auf zellulärer Ebene *in vitro* und *in vivo* zu erstellen und die zentralen regulatorischen Funktionen wichtiger involvierter Transkriptionsfaktoren darzulegen (Voigt, et al. 2017). In Folgendem sollen die ausschließlich durch meine Arbeit entstandenen Abbildungen und Kontributionen zu diesem Manuskript ausgeführt werden.

7.1.1 Eigenanteil der Publikation

Zunächst konnte ich in Figure 1E und 1F die zentrale Beteiligung von IL-1α und IL-23 bei der Induktion von IL-22 im murinem System illustrieren. So konnte ich in Splenozyten die durch Zugabe von Brustkarzinom (4T1)- oder Lungenkarzinom (Line-1)-Überständen induzierte Sekretion von IL-22 mittels anti-IL-1α- und anti-IL-23-blockierender Antikörper nahezu vollständig supprimieren. Die generierten murinen *in vitro* Daten sollten anschließend in humanen Ko-Kultur Experimenten validiert und die Übertragbarkeit der Erkenntnisse des murinen Systems auf den Menschen geprüft werden. Zunächst untersuchte ich deshalb, ob auch Tumorüberstände nicht-neoplastischer Zellen (*Human embryonic kidney 293 cells*, HEK293) die Sekretion von IL-22 aus humanen PBMC induzieren. Hierbei konnte ich demonstrieren, dass Überstände von HEK293 Zellen im Gegensatz zu humanen Brust- oder Lungen-karzinomüberständen nicht zur Produktion von IL-22 führen, was auf einen Tumorzell-spezifischen Mechanismus hindeutet (Figure S3A). Im nächsten Schritt sollte wie im murinen System die Involvierung der IL-1-Zytokine bei der Produktion von IL-22 analysiert werden. Mittels *enzyme-linked immunosorbent assay* (ELISA) untersuchte ich deshalb zunächst den Anteil der beiden Zytokine im Tumorüberstand und im Überstand humaner PBMC mit oder ohne Stimulation (Figure S3B – E). Nach Stimulation mit den jeweiligen Brust- oder Lungenkarzinomüberständen waren sowohl IL-1α als auch IL-1β im Überstand nachweisbar, was auf eine regulatorische Funktion beider Zytokine hindeutet. In Zusammenschau mit anderen Ergebnissen unseres Manuskripts lassen diese Erkenntnisse auf eine komplexe Signalkaskade schließen, welche schlussendlich zur IL-22 Produktion führt: Im Tumorüberstand befindliche Faktoren induzieren zunächst die Produktion der IL-1-Zytokine in humanen PBMC. In

einem nachgeschalteten Schritt kommt es wiederum durch auto- oder parakrine Stimulation unterschiedlicher Immunzellsubtypen (v.a. CD4+ T-Zellen) zur Ausschüttung von IL-22 (vgl. Abbildung 8). Weiterführende Experimente demonstrierten hierbei, dass v.a. sezerniertes IL-1β und weniger IL-1α entscheidend zur Induktion von IL-22 beiträgt (vgl. Figure S3F – I). Deshalb analysierte ich im nächsten Schritt die zelluläre Quelle von IL-1β in den von uns etablierten humanen *in vitro* Modellen. In Kongruenz mit der gängigen Literatur konnte ich mittels intrazellulärer *fluorescence-activated cell sorting (*FACS)-basierter Immunzell-phänotypisierung myeloide CD14+ Immunzellen als Hauptproduzenten von IL-1β identifizieren (Figure S4C). Um die in die IL-22 Ausschüttung involvierten molekularen Mechanismen noch weiter zu entschlüsseln und die Rolle des NLRP3-Inflammasoms diesbezüglich herauszuarbeiten, analysierte ich die Wirkung eines pan-Caspase-Inhibitors (Z-VAD) auf die IL-22 Produktion. Hierbei konnte ich eine dosisabhängige, signifikante Reduktion der IL-22 Sekretion nach Behandlung mit Z-VAD demonstrieren (Figure S5A, B; P. May, 2019). Abschließend sollte die Relevanz der *in vitro* Erkenntnisse auch im humanem Patientenkollektiv nachgewiesen werden. Mittels intrazellulärer FACS-basierter Methoden analysierte ich die Expression von IL-22 in primären humanen Lungenkarzinomgeweben jeweils im Vergleich zum gesundem Lungengewebe der Patienten. Die im Rahmen von Vorarbeiten (C. Voigt, 2017) generierten Daten wurden von mir ergänzt. Mittels der FACS-Analysen konnte ich eine signifikante Erhöhung der Anzahl IL-22 positiver Zellen im Tumor im Vergleich zum Normalgewebe demonstrieren (Figure S7A, S7B). Nach Generierung von Proteinlysaten des Tumorgewebes und des gesunden Kontrollgewebes analysierte ich außerdem die enthaltene Gesamtproteinmenge (pg/ml/mg) mittels ELISA. Lineare Regressionsanalysen (Pearson Korrelation) zeigten hierbei eine signifikante Korrelation zwischen der Gesamtproteinmenge von IL-22 und IL-1α.

Neben der Erstellung dieser Abbildungen leistete ich zu zahlreichen weiteren Datensätzen des Manuskriptes einen substantiellen Beitrag. Alle Daten, die zur Erstellung von Figure 2 (A – H) und Figure 3 (A – H) verwendet wurden, entstanden in Zusammenarbeit mit den genannten Ko-Autoren. Ebenso war ich an der Daten-erhebungen und der Erstellung aller weiteren Abbildungen der Supplementary Figures S3 – S8 beteiligt.

Mein Beitrag zu diesem Originalartikel und die unter meiner Leitung durchgeführte Revision des Manuskripts wurden mit einer geteilten Erstautorenschaft belohnt. Die Komplexität und der Umfang des Manuskriptes, sowie der hohe wissenschaftliche

Anspruch des Journals setzten eine Vielzahl weiterer Versuche voraus, die unter der Leitung der genannten Ko-Autoren durchgeführt wurden.

Die *supplementary Figures* können auf der Homepage von *Proceedings of the National Academy of Sciences* (PNAS) oder unter folgendem Link aufgerufen werden: http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1705165114/-/DCSupplemental

7.1.2 Originalartikel

Der Originalartikel „*Cancer cells induce interleukin-22 production from memory CD4+ T cells via interleukin-1 to promote tumor growth*“ (doi: 10.1073/pnas.1705165114) ist unter folgendem Link zu finden: https://www.pnas.org/content/114/49/12994.

PNAS

Cancer cells induce interleukin-22 production from memory CD4$^+$ T cells via interleukin-1 to promote tumor growth

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IL-22 has been identified as a cancer-promoting cytokine that is secreted by infiltrating immune cells in several cancer models. We hypothesized that IL-22 regulation would occur at the interface between cancer cells and immune cells. Breast and lung cancer cells of murine and human origin induced IL-22 production from memory CD4$^+$ T cells. In the present study, we found that IL-22 production in humans is dependent on activation of the NLRP3 inflammasome with the subsequent release of IL-1β from both myeloid and T cells. IL-1 receptor signaling via the transcription factors AhR and RORγt in T cells was necessary and sufficient for IL-22 production. In these settings, IL-1 induced IL-22 production from a mixed T helper cell population comprised of Th1, Th17, and Th22 cells, which was abrogated by the addition of anakinra. We confirmed these findings in vitro and in vivo in two murine tumor models, in primary human breast and lung cancer cells, and in deposited expression data. Relevant to ongoing clinical trials in breast cancer, we demonstrate here that the IL-1 receptor antagonist anakinra abrogates IL-22 production and reduces tumor growth in a murine breast cancer model. Thus, we describe here a previously unrecognized mechanism by which cancer cells induce IL-22 production from memory CD4$^+$ T cells via activation of the NLRP3 inflammasome and the release of IL-1β to promote tumor growth. These findings may provide the basis for therapeutic interventions that affect IL-22 production by targeting IL-1 activity.

interleukin-22 | interleukin-1 | inflammasome | cancer immunology | anakinra

IL-22 is a cytokine with tumor-promoting properties. It enhances tumor-cell proliferation, protects against apoptosis, and mediates the attraction of immunosuppressive immune cells and the release of pro- and antiinflammatory cytokines (1). IL-22 also promotes neoangiogenesis and epithelial-to-mesenchymal transition, which are hallmarks of cancer (1, 2). Unlike other cytokines, IL-22 is produced only by immune cells and binds to IL-22 receptor-1$^+$ (IL-22-R1$^+$) nonimmune cells (3). Strong evidence links IL-22 to colon cancer pathogenesis in both inflammatory and genetic colon cancer models (4–6). IL-22 drives the progression of hepatocellular carcinoma, potentially via accelerated tumor-cell proliferation (7, 8). The presence of IL-22–producing cells is linked to a more aggressive phenotype in a variety of cancer entities such as lung, breast, gastric, and skin cancer, indicating a more universal function of IL-22 in cancer progression (9–13). The source of IL-22 in these tumor entities varies, including innate immune cells and CD4$^+$ T cells (1, 14). In contrast, the mechanisms by which cancer cells or other cell populations within the tumor environment induce IL-22 production remain unaddressed.

Under physiological conditions and in certain inflammatory diseases such as psoriasis, IL-22 is mainly produced by T cells with smaller contributions from other immune populations (15, 16). IL-22 production is regulated by the transcription factors retinoic acid-related orphan receptor-γ (ROR-γ) and aryl hydrocarbon receptor (AhR) (15). Different cytokines have been reported to induce IL-22 production, but no data are available on how cancer cells regulate IL-22 production (15).

We demonstrate that tumor cells can induce IL-22 production directly from immune cells via IL-1. IL-1 induces the production of IL-22 in a memory CD4$^+$ Th cell population in mice and

Significance

IL-22 has been identified as a cancer-promoting cytokine, but its regulation in cancer tissue has not been addressed. Using both murine and human models, we demonstrate that cancer cells directly induce IL-22 production. We prove that interleukin-1β induced by inflammasome activation is critical for IL-22 production. IL-1β increased the activity of the IL-22 transcription factors in lineage-committed T cells. We show the existence of IL-22–producing Th1, Th17, and Th22 cells in tumor tissue of patients. Use of the clinically approved IL-1 receptor antagonist anakinra in vivo reduced IL-22 production and reduced tumor growth in a breast cancer model. These data provide the basis for therapeutic interventions, particularly using anakinra, aiming at limiting IL-22 production in patients with cancer.

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The authors declare no conflict of interest.

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Data deposition: Raw data and reagents will be made available upon reasonable request to the authors.

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humans. We show that reducing endogenous IL-1 activity in vivo, by administering anakinra, leads to diminished IL-22 production and subsequently reduced tumor burden in two different tumor models. In mice, dying tumor cells release IL-1α and initiate IL-22 production, but in humans, cancer cells trigger activation of the NLRP3 inflammasome and the release of IL-1β to regulate IL-22 in memory CD4$^+$ T cells. In patients, we found a strong correlation between the expression of IL-22–related genes and inflammasome activation, supporting the in vivo relevance of our findings. Our study unravels a shared mechanism of IL-22 induction across different cancer types. These findings identify IL-1 as a possible target to therapeutically modulate IL-22 production.

Results

IL-22 Production in Splenocytes Is Induced by Soluble Factors Released by Murine Breast and Lung Cancer Cell Lines. IL-22 is expressed in most cancer tissues studied so far, including breast and lung cancer (9, 10, 17). To investigate the source and the regulation of IL-22 production in these diseases, we first analyzed two murine syngeneic cancer models, 4T1 breast cancer and Line-1 lung cancer, for the presence of IL-22$^+$ cells by flow cytometry. We detected IL-22$^+$ cells with mononuclear cell morphology in spleen and tumor tissue in both models (Fig. 1 *A* and *B*). We hypothesized that these IL-22$^+$ cells are nontumor cells, e.g., infiltrating immune cells.

IL-22 was induced in splenocytes incubated with cell-free tumor cell-conditioned supernatants (Fig. 1 *C* and *D*).

Tumor-Derived IL-1α Drives IL-22 Production from Murine Splenocytes. To identify tumor-derived IL-22–inducing factors, we stimulated splenocytes with 4T1 and Line-1 cell supernatants and found 14 cytokines in the supernatants of the stimulated splenocytes (Fig. S1*A*). Of these 14 cytokines, IL-1α, IL-6, IL-23, IFN-γ, TNF-α, and G-CSF have been previously described as being involved in IL-22 induction (14, 18–20). IL-1β could not be detected. Testing for the effect of exogenously added cytokines detected only IL-1α and, to a lesser extent, IL-23 induced IL-22 from murine splenocytes (Fig. S1*B*). IL-1α and IL-23 were found in relevant amounts in both 4T1 and Line-1 cell lysates (Fig. S1 *C* and *D*) and in tumor-cell supernatants (Fig. S1 *E* and *F*). Stimulation of splenocytes with recombinant IL-1α and IL-23 alone or in combination dose-dependently mimicked stimulation with tumor-cell supernatants in terms of IL-22 induction (Fig. S1*G*). The addition of anti–IL-1α or anti–IL-23 neutralizing antibodies or both reduced IL-22 induction in splenocytes by tumor-cell supernatants (Fig. 1 *E* and *F*). Similarly, the addition of the IL-1 receptor (IL-1R) antagonist anakinra to tumor supernatants abrogated IL-22 induction in splenocytes (Fig. S1*H*). IL-22 induction was dependent upon IL-1R signaling, as stimulation of IL-1R–KO splenocytes with tumor supernatants or recombinant cytokines did not induce IL-22 production (Fig. 1 *G–I*).

Tumor Cells Induce IL-22 Production in Splenocytes via AhR and RORγt Signaling. To further dissect the mechanism of IL-22 induction by tumor cells, we sought to identify the transcription factors involved. Both AhR and RORγt have been described as playing a role in IL-22 production by immune cells under physiological conditions (21, 22). The addition of the AhR antagonist CH-223191 or the RORγt antagonist SR-2211 to splenocytes during stimulation with 4T1 and Line-1 cell supernatants significantly reduced IL-22 production. IL-22 production was blocked completely when both antagonists were added (Fig. 2 *A* and *B*). Moreover, treatment of tumor-bearing mice with repeated doses of CH-223191 decreased infiltrating IL-22$^+$ immune cells and the amount of IL-22 in tumor tissue in both the 4T1 and the Line-1 tumor models (Fig. S2 *A* and *B*), pointing toward a role for AhR signaling in IL-22 production in vivo as well.

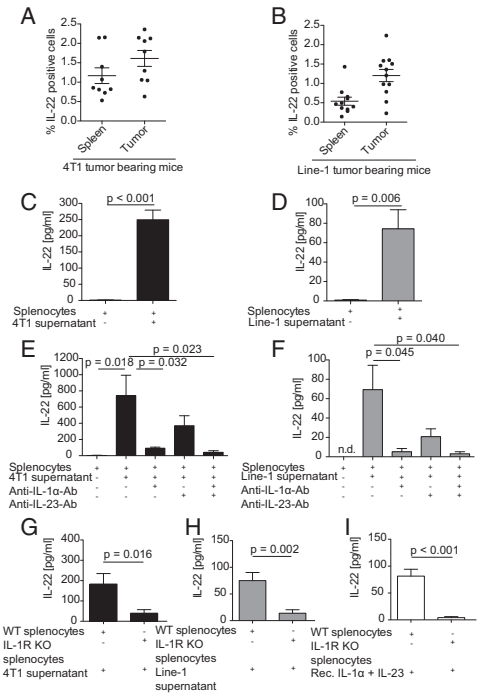

Fig. 1. Murine lung and breast cancer cell lines induce IL-22 from splenocytes via tumor-derived IL-1α. (*A* and *B*) Single-cell suspensions of 4T1 (*A*) and Line-1 (*B*) s.c. tumors were analyzed by flow cytometry for total intracellular IL-22 expression. Values in *A* and *B* represent pooled data of three independent experiments with three mice per group and four independent experiments with three mice per group, respectively. (*C* and *D*) Splenocytes (2×10^6/mL) were stimulated with 50% cell-free 4T1 (*C*) or Line-1 (*D*) tumor-cell supernatant for 6 d. Mean values from five independent experiments are shown. (*E* and *F*) Splenocytes were stimulated with 4T1 (*E*) or Line-1 (*F*) tumor-cell supernatant in the presence or absence of anti–IL-1α or anti–IL-23 blocking antibodies (2.5 μg/mL) or both for 6 d. The mean values of a minimum of four independent experiments are shown. (*G–I*) Wild-type or IL-1R knock-out splenocytes were stimulated with 4T1 supernatant (*G*), Line-1 supernatant (*H*), or 20 ng/mL recombinant IL-1α and IL-23, respectively (*I*). Values in *G–I* represent pooled data of two independent experiments with four to seven mice per group. IL-22 production was quantified by ELISA (*C–I*). Error bars represent the SEM; *P* values from a two-sided Student's *t* test are shown; n.d., not detectable; rec., recombinant.

Murine Tumor Cells Induce IL-22 Production from Memory CD4$^+$ T Cells. Based on the literature, T cells are a major source of IL-22 in mice and humans (14). We hypothesized that, if T cells are the major source of IL-22 in our system, IL-22 production should be conserved in the CD3$^+$ and CD4$^+$ splenocyte fraction while being reduced in the CD3- or CD4-depleted fraction. Purified CD3$^+$ T cells secreted IL-22 in comparable amounts to splenocytes when stimulated with tumor-cell supernatant. In contrast, IL-22 production was significantly lower in the CD3-depleted fraction (Fig. S2 *C* and *D*). Similarly, purified CD4$^+$ T cells but not CD4-depleted splenocytes produced IL-22 when stimulated with tumor-cell supernatants or with recombinant IL-1α and IL-23 (Fig. 2 *C* and *D*). To further characterize the source of

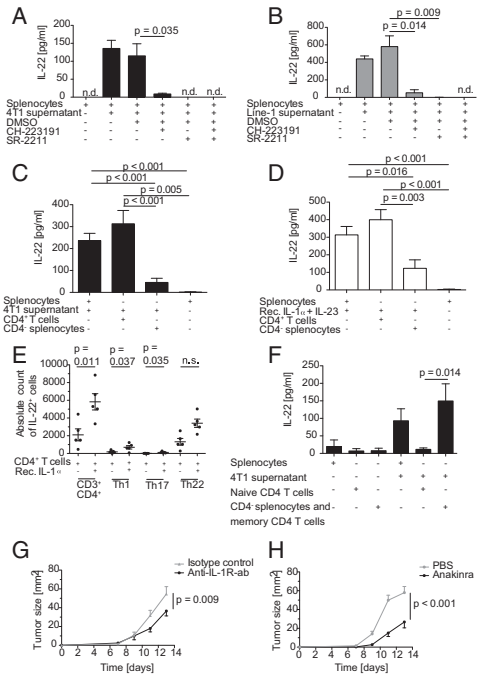

Fig. 2. IL-22 is secreted from memory CD4$^+$ T cells in an AhR- and RORγt-dependent manner, and in vivo neutralization of IL-1 reduces tumor growth. (*A* and *B*) Splenocytes (2×10^6/mL) were stimulated with 4T1 supernatant (*A*) or Line-1 supernatant (*B*) in the presence or absence of 10 μM CH-223191 (AhR antagonist) or 5 μM SR-2211 (RORγt antagonist) for 6 d. Values in *A* are the mean of three different experiments performed in triplicate. Values in *B* are representative of five different experiments performed in triplicate. IL-22 production was quantified by ELISA. (*C* and *D*) MACS-enriched CD4$^+$ T cells and CD4$^-$ splenocytes or total splenocytes were stimulated with 4T1 tumor supernatant (*C*) or 100 ng/mL recombinant IL-1α and IL-23 (*D*) for 6 d. Mean values of a minimum of four independent experiments are shown. (*E*) MACS-enriched CD4$^+$ T cells were stimulated with 20 ng/mL recombinant IL-1α for 4 d. IL-22 production by Th1 (CD3$^+$CD4$^+$IFN-γ$^+$), Th17 (CD3$^+$CD4$^+$IFN-γ$^-$IL-17$^+$), and Th22 (CD3$^+$CD4$^+$IFN-γ$^-$IL-17$^-$) T cells was analyzed by flow cytometry (data represent two independent experiments with five different mice). (*F*) MACS-enriched naive CD4$^+$ T cells and CD4$^-$ splenocytes including CD4$^+$ CD44$^+$ memory T cells were stimulated with 4T1 supernatant for 6 d. Mean values of three different experiments with eight replicates of supernatants are shown. (*G*) BALB/c mice bearing 1.25×10^5 4T1 tumor cells s.c. ($n = 10$ mice per group) were treated i.p. with 300 μg anti-mouse IL-1R antibody or isotype control every second day beginning on day 0. (*H*) C57BL/6 mice were injected s.c. in the right flank with 2.5×10^5 E0771 tumor cells ($n = 15$ mice per group). Mice were treated with 1 mg anakinra or PBS i. p. every day beginning on day 0. In *A*–*F* error bars represent the SEM, and *P* values by two-sided Student's *t* test are shown. In *G* and *H*, statistical significance was analyzed by two-way ANOVA with correction for multiple testing; n.d., not detectable; n.s., not significant; rec., recombinant.

IL-22 within the CD4$^+$ T cell population, we stimulated CD4$^+$ splenocytes with IL-1α and analyzed their phenotype by flow cytometry. Most IL-22$^+$ cells were CD3$^+$CD4$^+$ and consisted of a mixed Th1, Th17, and Th22 cell population (Fig. 2*E*). These phenotype findings were supported by analyzing the supernatants for the presence of the respective prototypical cytokines, such as IL-17 and IFN-γ. IFN-γ and IL-17 were cosecreted in high amounts (Fig. S2 *E* and *F*). The IFN-γ and IL-17 production of T cells was also IL-1α dependent, as the induction of either cytokine could be abrogated by the IL-1R antagonist anakinra (Fig. S2 *G* and *H*).

In tumor-bearing mice, we could identify both IL-22–producing CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cells in tumor tissue (Fig. S2 *I* and *J*). When mice were treated with the AhR antagonist, the amount of IL-22 production was reduced in these T cell fractions (Fig. S2*K*).

CD4$^+$CD44$^+$ memory T cells were identified as the target population for IL-1α, as IL-22 induction was detected only in this fraction but not in naive CD4$^+$ T cells or CD4$^-$ cells (Fig. 2*F*).

Blocking of IL-1 Signaling Reduces Tumor Progression and Production of IL-22$^+$ Cells in Vivo. To confirm our in vitro findings and the relevance of the identified pathway for tumor progression, we tested the impact of IL-1 blockade on tumor growth and IL-22 production in vivo. We used both a neutralizing IL-1R antibody and the soluble IL-1R antagonist anakinra. Tumor progression, as evidenced by tumor growth, was reduced when 4T1 tumor-bearing mice were treated with IL-1R antibody (Fig. 2*G*). Growth reduction was paralleled by reduced IL-22 production, as analyzed by flow cytometry, confirming the dependence of IL-22 production on IL-1 (Fig. S2*L*). Similarly, when we treated mice bearing the E0771 breast cancer model with anakinra, we again found a striking retardation of tumor growth (Fig. 2*H*). As seen in the 4T1 model, IL-22 production was again reduced when IL-1 activity was inhibited (Fig. S2*M*). These findings highlight the relevance of the IL-1–IL-22 pathway for cancer progression and point toward the potential use of approved IL-1–antagonizing agents such as anakinra for cancer therapy.

Tumor Cell-Derived Factors from Human Breast and Lung Cancer Cells Induce IL-22 Production from Peripheral Blood Mononuclear Cells. Based on the observations in mice, we next asked whether the observed induction of IL-22 production in immune cells by breast and lung cancer cells would also occur in human cells. Human peripheral blood mononuclear cells (PBMCs) from healthy donors were stimulated with tumor-conditioned supernatants of three human breast cancer (MCF7, CAMA-1, and MDAMB231) and three human lung cancer (A549, HCC827, and H1339) cell lines. Supernatants from both cancer cell types induced IL-22 production in human PBMCs, and this induction was attributable to soluble factors (Fig. 3 *A* and *B*). In contrast, stimulation of PBMCs with non–tumor-cell supernatant (from HEK293 cells) failed to lead to IL-22 production (Fig. S3*A*).

Tumor Cell-Derived IL-1α and Tumor Cell-Induced IL-1β Lead to IL-22 Production in Human PBMCs in an AhR- and RORγt-Dependent Manner. To further investigate the mechanism of IL-22 induction by cancer cells in human PBMCs, we added the IL-1R antagonist anakinra to the conditioned supernatants of breast and lung cancer cell lines. Anakinra blocked IL-22 induction in PBMCs stimulated with breast and lung cancer cell supernatants in a similar fashion (Fig. 3 *C* and *D*). In tumor-cell supernatants, we found IL-1α (but not IL-1β) in two of the human cell lines, H1339 and MDAMB231 cells (Fig. S3 *B* and *C*). IL-1α was also induced after stimulation with supernatants except for the H1339 supernatant (Fig. S3 *B* and *C*). Incubation of human PBMCs with any of the tumor-cell supernatants tested induced IL-1β production (Fig. S3 *D* and *E*). For all human cell lines analyzed, IL-1β was the main driver of IL-22 induction in PMBCs, as anti–IL-1β but not anti–IL-1α antibodies antagonized IL-22 induction in HCC827, H1339, CAMA-1, and MDAMB231 cell lines (Fig. S3 *F*–*I*). At the transcription factor level, IL-22 induction was dependent on both AhR and RORγt signaling in PBMCs (Fig. 3 *E* and *F* and Fig. S4 *A* and *B*).

Human Breast and Lung Cancer Cells Activate the NLRP3 Inflammasome to Induce IL-22 from Memory CD4$^+$ T Cells. We next addressed the cellular sources of IL-1β within the PBMCs by flow cytometry and

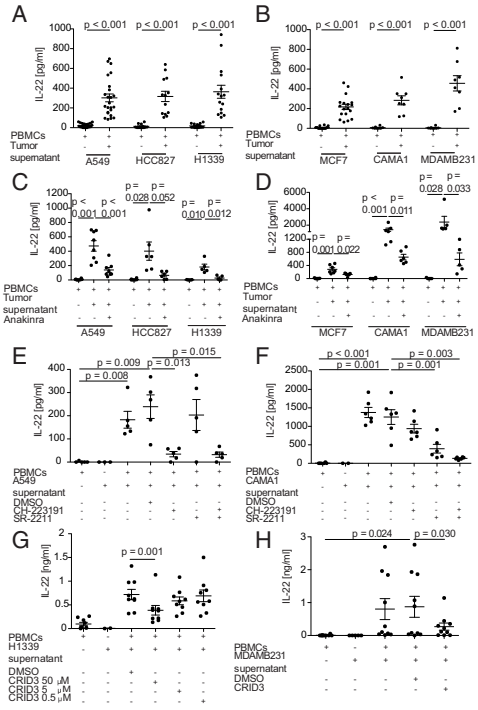

Fig. 3. Human lung and breast tumor supernatants activate the NLRP3 inflammasome to release IL-1 and induce IL-22 in PBMCs from healthy donors in an AhR- and RORγT-dependent manner. (*A–D*) PBMCs (2×10^6/mL) were stimulated with 50% cell-free human lung cancer cell (A549, HCC827, H1339) conditioned supernatant (*A*) or breast cancer cell (MCF7, CAMA-1, MDAMB231) conditioned supernatant (*B*) for 6 d in the presence or absence of anakinra (500 ng/mL) (*C* and *D*). (*E* and *F*) PBMCs were stimulated with A549-conditioned (*E*) or CAMA-1–conditioned (*F*) tumor-cell supernatant for 6 d in the presence or absence of 10 μM CH-223191 or 5 μM SR-2211. (*G* and *H*) PBMCs were stimulated with H1339-conditioned (*G*) or MDAMB231-conditioned (*H*) tumor-cell supernatant for 6 d in the presence or absence of 50, 5, or 0.5 μM (H1339) or 50 μM (MDAMB231) CRID3. IL-22 production was quantified by ELISA (*A–H*). n = 8–24 different donors in *A* and *B*, 5–8 different donors in *C* and *D*, 5–6 different donors in *E* and *F*, 9 different donors in *G*, and 10 different donors in *H*. Each dot in the graphs represents one donor. Error bars represent the SEM; *P* values from a two-sided Student's *t* test are shown.

detected IL-1β production by myeloid cells and to a lesser extent by $CD4^+$ T cells after stimulation with tumor supernatants (Fig. S4*C*). To further dissect the mechanism responsible for IL-1β production, we used the specific NLRP3 inhibitor, the cytokine release inhibitory drug CRID3, and the pan-caspase inhibitor Z-Vad. The addition of CRID3 and Z-Vad abolished IL-22 production in a concentration-dependent manner (Fig. 3 *G* and *H* and Fig. S5 *A* and *B*), suggesting the involvement of inflammasome activation by the tumor cells.

To identify the cell type responsible for IL-22 production upon the addition of tumor cell-conditioned supernatants to PBMCs, we analyzed their phenotype by flow cytometry. Most $IL\text{-}22^+$ cells were $CD3^+CD4^+$ and consisted of a mixed Th1, Th17, and Th22 cell population (Figs. S4 *D* and *E* and S5 *C* and *D*). We purified and stimulated $CD4^+$ T cells with lung and breast cancer cell supernatants. Secretion of IL-22 by $CD4^+$ T cells was comparable to that from whole PBMCs, but IL-22 production was almost absent in the CD4-depleted fraction (Figs. S4 *F* and *G* and S5 *E* and *F*). Similarly, stimulation of $CD4^+$ T cells, but not of CD4-depleted PBMCs, with recombinant IL-1α or IL-1β led to IL-22 production (Fig. S4*H*), indicating that the $CD4^+$ T cell fraction is the target of IL-1 and the source of IL-22 production. To further characterize the T cell population involved, we isolated naive and memory $CD4^+$ T cells. We found that isolated memory $CD4^+$ T cells were proficient IL-22 producers in our settings. Naive $CD4^+$ T cells did not produce IL-22, whereas the naive $CD4^+$ T cell-depleted fraction retained their ability to induce IL-22 (Fig. S5*G*). IL-22 induction by A549 tumor supernatant was still seen in the purified $CD4^+$ T cell population and was dependent on the activation of the NLRP3 inflammasome (Fig. S5*H*).

This mixed phenotype of different Th lineages was further confirmed by analysis of supernatants after stimulation of PBMCs with tumor-conditioned supernatants. IFN-γ and IL-17 cosecretion was found in all cultures analyzed, supporting the presence of Th1, Th17, and Th22 cells in the culture (Fig. S6 *A–D*). The production of IL-22 appears to be dependent on these subpopulations, as addition of the IL-1R antagonist anakinra to the PBMCs incubated with tumor-cell supernatants blocked the induction of both IFN-γ and IL-17 in addition to blocking IL-22 production (Fig. S6 *E–H*). These results indicate that tumor cells can induce cytokine production from Th1, Th17, and Th22 cells in an IL-1R–dependent manner via activation of the NLRP3 inflammasome.

Th Cells Are the Main Source of IL-22 in Primary Human Lung and Breast Cancer Tissue. To confirm the existence of IL-22–producing Th cell populations in primary human breast and lung cancer, we next analyzed tumor samples of patients with lung (n = 23) and breast (n = 11) cancer for the presence of these cells by flow cytometry. In lung cancer samples 0.58% and in breast cancer samples 0.23% of the mononuclear cell fraction expressed IL-22 (Figs. S7 *A* and *B* and S8 *A* and *B*). Among these $IL\text{-}22^+$ cells in lung cancer samples, the main fraction, accounting for 50% of these cells, was of a Th1 phenotype, followed by Th22 and Th17 phenotypes (14% and 6%, respectively) (Fig. S7*C*). Expression of IL-22 in lung cancer tissue was confirmed in protein lysates of the same tumor samples (Fig. S7*D*). IL-22 was found at higher levels in lung cancer than in matched nontumor tissue from the same patient (Fig. S7 *B* and *D*). To corroborate the link between IL-22 production and IL-1, we next correlated protein levels of both cytokines. We found a significant correlation between IL-22 and IL-1α, compatible with the dependence of IL-22 production on the presence IL-1 in tumor tissue from patients (Fig. S7*E*). Expression of IL-22 could also be confirmed in breast cancer tissue (n = 7) (Fig. S8*D*). There, in line with our in vitro findings, we could confirm a predominant expression of IL-22 by $CD4^+$ T cells consisting of Th1 (41%), Th17 (9%), and Th22 (23%) cells (Fig. S8*C*).

IL-22 Expression and Inflammasome Activation Correlate in Human Lung and Breast Adenocarcinoma. To further link inflammasome activation with IL-22 production, we next analyzed expression data from two clinical cohorts of patients with lung (n = 80) or breast (n = 45) cancer (23, 24). Thirty-three transcripts related to the IL-22 pathway were arbitrarily selected (Fig. S9*A*). Lung and breast samples were hierarchically clustered to these 33 transcripts, and transcripts that clustered together and were closely related with *IL22* were further analyzed by hierarchical clustering to identify their power to discriminate normal from cancer tissues (Fig. S9 *B* and *D*). The inflammasome-related genes enabled differentiation between cancer and noncancer tissue in lung cancer but not in breast cancer (Fig. S9 *B* and *D*). When lung and breast cancer tissues were analyzed separately, excluding normal tissues, we found a clear correlation between inflammasome- and IL-22–related

genes, further underscoring the relationship between IL-22 production and inflammasome activation (Fig. S9 *C* and *E*).

Discussion

Our study describes a mechanism by which cancer cells induce IL-22 production from $CD4^+$ T cells in mice and in humans in an AhR- and RORγt-dependent manner. In mice, IL-22 production is dependent on IL-1α release by cancer cells. IL-22 content in tumor tissue and tumor growth are reduced when IL-1 is neutralized in two different breast cancer models. Human cancer cells induce IL-22 through the activation of the NLRP3 inflammasome, resulting in IL-1β release. In patients, the degree of inflammasome activation correlates with IL-22 content in human breast and lung cancer samples.

A role for IL-22 in cancer development and progression has been recognized in several epithelial cancers, including breast and lung cancer (1, 9). When released by immune cells, IL-22 can act on cancer cells to promote tumor growth, aggressiveness, and treatment resistance (1, 25). However, no study has yet investigated the mechanism by which IL-22 production is induced or which immune cells are able to produce IL-22 in the tumor environment. Our findings provide evidence that cancer cells affect memory $CD4^+$ T cells to express and release IL-22 in an IL-1–dependent manner and that this shared mechanism promotes tumor growth.

IL-1α and IL-1β are two cytokines with shared signaling via IL-1R but with a different biology (26). IL-1β is a driver of IL-22 production by immune cells and by Th17 cells in particular (27–29). In our study, IL-1R signaling was central to cancer cell-driven IL-22 production, but the mediating IL-1 family members differed between species. In mice, IL-1α was the main inducer of IL-22 production and was detected in the supernatants and protein lysates of cancer cells. IL-1α is mostly cell-associated in viable cells but may be released from dying tumor cells (30, 31). In addition, IL-1α can induce its own release from immune cells, further enhancing its effects on $IL\text{-}1R^+$ cells, as previously described (32).

IL-1 is detectable in human breast, colon, lung, and head and neck cancers and in melanoma. Its detection is typically associated with a worse prognosis (17). In line with these findings, inhibition of endogenous IL-1 activity by administering anakinra reduces both the extent of metastasis and tumor burden (18). Comparable effects are also known for the NLRP3 inflammasome (33). Our findings link endogenous IL-1 activity to IL-22 induction in two different breast cancer models.

In contrast to the murine system, in human cancer cell lines cocultured with PBMCs, IL-1β is the main inducer of IL-22 and is induced in PBMCs. The specific NLRP3 inhibitor CRID3 abolished both IL-1β and IL-22 production in whole PBMCs and in purified $CD4^+$ memory T cells, indicating that NLRP3 is required for inflammasome activation. A mechanism of NLRP3 inflammasome activation could be the release of IL-1α precursor or uric acid from dying tumor cells. These act on immune cells and activate the NLPR3 inflammasome (34–37). Release of ATP from tumor cells or tumor cell-derived nucleic acids may also result in NLRP3 activation and IL-1β release (38, 39).

We have identified memory $CD4^+$ T cells as a primary target of the released IL-1β for IL-22 induction. Mechanistically, IL-1β could activate a preexisting pool of Th1, Th17, and Th22 cells for IL-22 production (40). Alternatively, there is evidence that IL-1β can drive the differentiation of memory T cells to these Th cell lineages (41, 42).

The transcription factor RORγt is required for Th17 and Th22 polarization, and the transcription factor AhR is additionally required for Th22 differentiation in mice (43–45). Similarly, RORγt and AhR are involved in IL-22 production (46, 47). Our findings show that both transcription factors are also required for IL-22 production in the setting of cancer cell-induced IL-22 production.

The cellular source of IL-22 in the tumor microenvironment varies according to the tumor entity and the species studied (1, 5). In the present study, we demonstrate that murine and human breast and lung cancer cells induce IL-22 production from a mixed population of Th1, Th17, and Th22 cells in an IL-1–dependent manner. Our findings are compatible with studies that have identified Th1 and Th17 cells in breast and lung cancer (48–50). The presence of these cells has been reported to correlate with worsened clinical outcomes (49, 51, 52). We could identify a positive correlation between inflammasome activation and IL-22 production in tumor tissue specimens from two patient cohorts with lung and breast cancer, respectively.

In summary, our study describes a previously unrecognized mechanism by which cancer cells can induce IL-22 in T cells. It provides a link between the widely described expression and function of IL-22 in cancer and its cellular source (Fig. S10). On another line, we provide pathophysiological insights into the effect of clinical IL-1 blockade recently described in lung cancer (53). The availability of clinically approved IL-1–antagonizing agents such anakinra and its favorable safety profile place clinical testing for tumor indications within reach. Clinical trials with anakinra in patients with breast cancer are under way, so far showing promising preliminary data (54). Our findings add to the rationale for developing therapeutic interventions targeting the IL-1–IL-22 axis.

Materials and Methods

Mice. All animal experiments were approved by the local regulatory agency (Regierung von Oberbayern) or by the Veterinary Administration of the Prefecture of Western Greece (protocol approval no. 118018/578) and adhered to the NIH guidelines for the care and use of laboratory animals.

Mouse Tumor Models and Treatment of Mice. Five-week-old BALB/c mice were injected s.c. in the right flank with 1.25×10^5 4T1 or 5×10^5 Line-1 tumor cells, and C57BL/6 mice were injected s.c. in the right flank with 2.5×10^5 E0771 tumor cells and were treated as indicated.

Patient Samples. The tissue samples of non-small cell lung cancer (NSCLC) and corresponding clinical data used in this study were provided by the Biobank under the administration of Human Tissue and Cell Research (HTCR) Foundation at University Hospital, Ludwig Maximilian University, Munich (LMU Munich). The framework of the HTCR includes obtaining written informed consent from all patients with lung cancer and has been approved by the Ethics Committee of the Medical Faculty, LMU Munich (no. 025-12) and by the Bavarian State Medical Association. All operations of Biobank are certified according to ISO 9001:2008. Written informed consent was obtained from all patients with breast cancer before collection of specimens, in line with the respective institutional policies and in accordance with to the Declaration of Helsinki. Tumor specimens were obtained from patients undergoing clinically indicated surgery. Ethical approval was obtained from the Ethics Committee of the Medical Faculty, LMU Munich (reference nos. 220-15 and 249-15).

Cytokine Secretion Assays. Splenocytes, purified T cells, and PBMCs were cultivated as indicated (see *SI Materials and Methods* and figure legends for details).

Flow Cytometry. Flow cytometry was performed according to standard protocols as indicated (see *SI Materials and Methods* for details).

Statistics. FlowJo V9.2 software (TreeStar) was used for analysis of FACS datasets. Statistics were calculated with GraphPad Prism software 5.0. Differences between experimental conditions were analyzed using the unpaired two-tailed Student's *t* test. The Mann–Whitney *U* test was used to compare data points from individual mice. A paired two-tailed Student's *t* test was used when comparing experimental conditions for individual human donors. Statistical significance was analyzed by two-way ANOVA with correction for multiple testing in case of tumor growth curves. P values < 0.05 were considered significant.

Data Availability. All data supporting this paper are attached. Raw data and reagents will be made available upon reasonable request to the authors.

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7.2 Interleukin-37 monomer is the active form for reducing innate immunity

Im Rahmen des von uns veröffentlichten Manuskriptes *„Interleukin-37 monomer is the active form for reducing innate immunity“* waren wir in der Lage, mittels biophysikalischer, biochemischer und biologischer Methoden darzulegen, dass die monomere Form von IL-37 im Gegensatz zur dimerisierten Form zu einer verstärkten Suppression unterschiedlicher angeborener, inflammatorischer Kaskaden führt (Eisenmesser, et al. 2018).

7.2.1 Eigenanteil der Publikation

Mein Beitrag zu der genannten Publikation konzentriert sich vor allem auf die biologische Bestätigung der gesammelten biophysikalischen Daten. In Figure 4C und 4D stellte ich zunächst die charakteristische, parabolische Wirkungskurve von IL-37 in einem murinen *in vitro* Modell dar. Durch Behandlung der monozytären J774A.1-Zelllinie mit LPS und Nigericin sowie unterschiedlichen IL-37-Konzentrationen konnte ich die dosisabhängige Inhibierung der Sekretion von IL-1β und IL-6 aufzeigen.
Im Rahmen von Figure 5A und 5B untersuchte ich die Effekte von unterschiedlichen strukturellen Modifikationen des IL-37 Proteins auf dessen Funktion. Einzelne N- bzw. C- terminale Trunkierungen des Proteins schienen die anti-inflammatorischen Eigenschaften nur wenig zu beeinflussen (Figure 5A). Die inhibitorischen Effekte von IL-37 zeigten sich jedoch durch Modifikation beider Enden (IL-37, 53-206) geringfügig verringert. Durch Austausch einzelner Aminosäuren, welche die Dimerisierung des IL-37 Proteins verhindern (Y85A, D73K), ließen sich die anti-inflammatorischen Effekte jedoch signifikant verstärken (Figure 5B). Diese Resultate bestätigten die zuvor generierten biophysikalischen Daten (Figure 3).

Mein Beitrag zu diesem Originalartikel wurden mit einer Ko-Autorenschaft belohnt. Die *supplementary Figures* können auf der Homepage von *Proceedings of the National Academy of Sciences* (PNAS) oder unter folgendem Link aufgerufen werden: https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1819672116/-/DCSupplemental

7.2.2 Orginalartikel

Der Originalartikel *„Interleukin-37 monomer is the active form for reducing innate immunity“* (doi: 10.1073/pnas.1819672116) ist unter folgendem Link zu finden: https://www.pnas.org/content/116/12/5514

Interleukin-37 monomer is the active form for reducing innate immunity

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Interleukin-37 (IL-37), a member of the IL-1 family of cytokines, is a fundamental suppressor of innate and acquired immunities. Here, we used an integrative approach that combines biophysical, biochemical, and biological studies to elucidate the unique characteristics of IL-37. Our studies reveal that single amino acid mutations at the IL-37 dimer interface that result in the stable formation of IL-37 monomers also remain monomeric at high micromolar concentrations and that these monomeric IL-37 forms comprise higher antiinflammatory activities than native IL-37 on multiple cell types. We find that, because native IL-37 forms dimers with nanomolar affinity, higher IL-37 only weakly suppresses downstream markers of inflammation whereas lower concentrations are more effective. We further show that IL-37 is a heparin binding protein that modulates this self-association and that the IL-37 dimers must block the activity of the IL-37 monomer. Specifically, native IL-37 at 2.5 nM reduces lipopolysaccharide (LPS)-induced vascular cell adhesion molecule (VCAM) protein levels by ∼50%, whereas the monomeric D73K mutant reduced VCAM by 90% at the same concentration. Compared with other members of the IL-1 family, both the N and the C termini of IL-37 are extended, and we show they are disordered in the context of the free protein. Furthermore, the presence of, at least, one of these extended termini is required for IL-37 suppressive activity. Based on these structural and biological studies, we present a model of IL-37 interactions that accounts for its mechanism in suppressing innate inflammation.

interleukin | inflammation | dimer | innate immunity

The discovery of IL-1 marked a revolution in biology with the identification of a single highly active protein that could induce inflammation at picamolar concentrations (1). In many ways, the discovery of IL-37 has led to a similar revolution whereby IL-37 can limit or even prevent a broad spectrum of inflammatory models now termed innate immunity. Specifically, IL-37 can reverse inflammation, diminish multiple phenotypes related to cancer, and even reverse age-related phenotypes (2–6). Much of the molecular details of IL-37 signaling has been surmised from animal models and cellular studies that include colocalization studies, which suggest that IL-37 engages both the IL-18Rα receptor and the previously orphaned IL-1R8 receptor (7–10). The elegance of this proposed signaling mechanism is that IL-37 could both block the proinflammatory-induced response by IL-18 and simultaneously signal to promote an antiinflammatory response. However, the molecular details of IL-37 remain elusive as IL-37 exhibits several unique features relative to other IL-1 family members, which include the formation of a dimer and the presence of extended termini.

Unlike other members of the IL-1 family that are monomeric, IL-37 self-associates with nanomolar affinity (11, 12) and comprises longer extended termini relative to other family members. ILs from different families have been shown to dimerize with a spectrum of functional consequences, which range from IL-10 family members that are fully active as swapped dimers (13, 14) to chemokines, such as IL-8 that engage their receptors primarily as monomers (15–17). Indeed, the recent crystal structure of IL-37 by Ellisdon et al. (12) has afforded the engineering of IL-37 mutant forms that disrupt dimer formation and simultaneously lead to higher IL-37 activities. However, the activity of these IL-37 mutant forms were compared well below their determined self-association affinities where dimer formation would be expected to be negligible, suggesting the presence of cellular determinants that further modulate dimerization analogous to an array of molecules that modulate chemokine dimerization (18). Considering the effectiveness of IL-1 blocking therapies already in use today (19), further elucidating the activities of these monomeric IL-37 forms and the molecular determinants of IL-37 activity may open a window to new therapies that utilize variations of IL-37 to block acute inflammation and potentially cancer progression. Thus, here we sought to address the solution behavior of IL-37 and its monomeric forms, determine whether the higher activities of IL-37 monomeric versions are a more general phenotype that would further support an inhibitory role for the IL-37 dimer, and determine the role of the extended IL-37 termini.

Significance

IL-37 is the newest member of the IL-1 family of cytokines that elicit diverse activities integrally related to the innate immune responses and inflammation. Whereas most members elicit proinflammatory responses; in contrast, IL-37 broadly down-regulates inflammation and exists in a unique monomer/dimer equilibrium. Experimental and theoretical analyses show that monomeric IL-37 results in more effective suppression of inflammatory markers on multiple cell types compared with native IL-37, whereas the IL-37 dimer functions to block the activity of the monomer. Considering the emerging efficacy of antagonists and antibodies that specifically target IL-1 in human disease, identifying the active form of IL-37 may lead to the clinical development of recombinant IL-37 to down-regulate inflammation.

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The authors declare no conflict of interest.

Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.wwpdb.org (PDB ID code 6NCU) and in Biological Magnetic Resonance Data Bank (BMRB accession no. 27729).

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Results and Discussion

Only Mutations at the IL-37 Dimer Interface Specifically Disrupt Dimer Formation. The biological form of secreted IL-37 comprises residues 46–218 (IL-37$^{46–218}$), and terminal truncations together with NMR analysis described below identified a stable core of IL-37$^{53–206}$ amenable to crystallization and subsequent structure determination. Whereas the recent X-ray crystal structure of a similar construct of IL-37$^{50–206}$ was solved with the proposed dimer interface formed between IL-37 monomers within different asymmetric units (12), IL-37$^{53–206}$ crystallized here was in the $P3_121$ space group where each dimer was visible within each asymmetric unit [Protein Data Bank (PDB) accession number 6NCU]. Thus, despite crystallization within a completely different space group and relatively low resolution of these crystals (Table 1), density across symmetry mates clearly identifies the same dimer interface as this recent IL-37 structure (Fig. 1*A*). Specifically, Asp73 in one monomer forms a stabilizing ionic bond with Lys83 in another monomer, and Tyr85 forms a hydrophobic cluster with Val71 and Ile78 across this interface. As expected, mutation of either IL-37^{D73K} (D73K) or IL-37^{Y85A} (Y85A) results in stable monomeric forms as assessed by size-exclusion chromatography, whereas a control mutation of IL-37^{D82K} retains dimer formation (Fig. 1*B*). Thus, IL-37 dimers can be converted into IL-37 monomers by specific point mutations to the dimer interface. NMR studies were also undertaken to probe the solution behavior of IL-37 and characterize the extended termini of IL-37 that are longer than other IL-1 family members.

The IL-37 Termini Are Disordered, Whereas the Dimer Interface Is Highly Ordered. To address the solution behavior of IL-37 and provide a foundation for further comparative studies of the monomeric versions of IL-37, we exploited the high sensitivity of NMR. The WT biologically active form of IL-37$^{46–218}$ gives rise to a well-dispersed 2D-heteronuclear single quantum coherence (HSQC) spectrum that is indicative of a well-folded protein (Fig. 2*A*). However, a significant overlap of highly intense peaks within the center of the spectrum is suggestive of unfolded regions, which we hypothesized are due to the presence of disordered termini. To test this hypothesis, we truncated both termini to the smallest soluble recombinant protein of IL37$^{53–206}$, which

Table 1. Structural statistics for the IL-37 crystal structure

Data collection	
Space group	$P3_121$
Cell dimensions	
a, *b*, *c* (Å)	67.50, 67.510, 142.74
α, β, γ (Å)	90.0, 90.0, 120.0
Resolution (Å)	3.5
R_{merge} (%)	9.0
$I/\sigma I$	16.6
Completeness (%)	99
Redundancy	19
Refinement	
Resolution (Å)	54.1–3.5
Number of reflections	5,091
R_{work}/R_{free}	34.0 (39.9)*
Number of atoms	2385
Protein	2385
B-factors	108.8
rms deviations	
Bond lengths (Å)	0.003
Bond angles (°)	0.0612
Ramachandran	
Favored (%)	83.8
Allowed (%)	12.84
Outliers (%)	3.4
Clash score	12

*Values in the parenthesis refer to the highest-resolution shell.

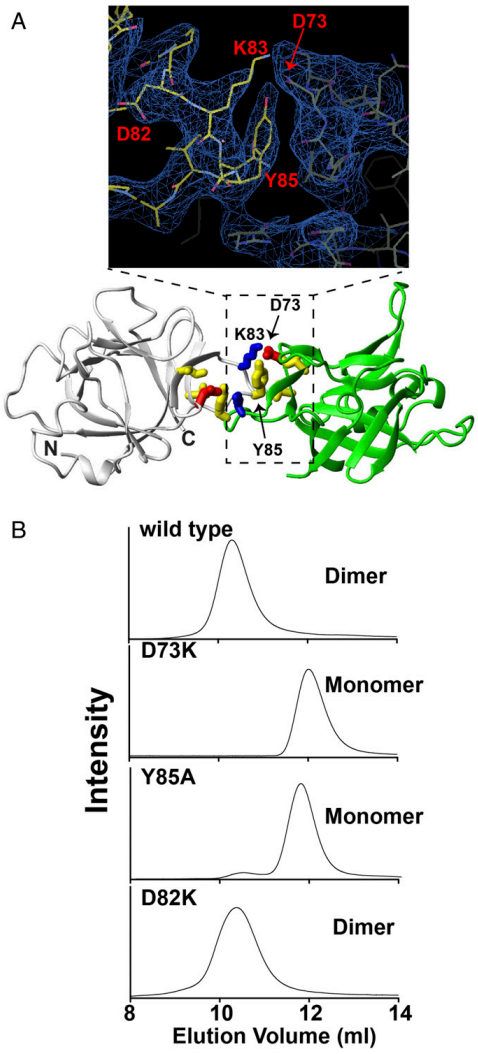

Fig. 1. IL-37 crystal structure and analytical sizing of IL-37 point mutations. (*A*) Electron density of the IL-37$^{53–206}$ dimer interface (*Top*) and key interactions (*Bottom*). These include an ionic interaction among D73 (red) and K83 (blue) and Y85 mediated hydrophobic interactions (yellow). (*B*) Analytical size-exclusion chromatography (Superdex 75) of wild-type (WT) IL-37 and monomer-inducing point mutations D73K and Y85A along with a control mutation IL-37^{D82K}. Analytical size exclusion was conducted in the context of the mature IL-37 (residues 46–218 with the WT sequence and the single point mutations).

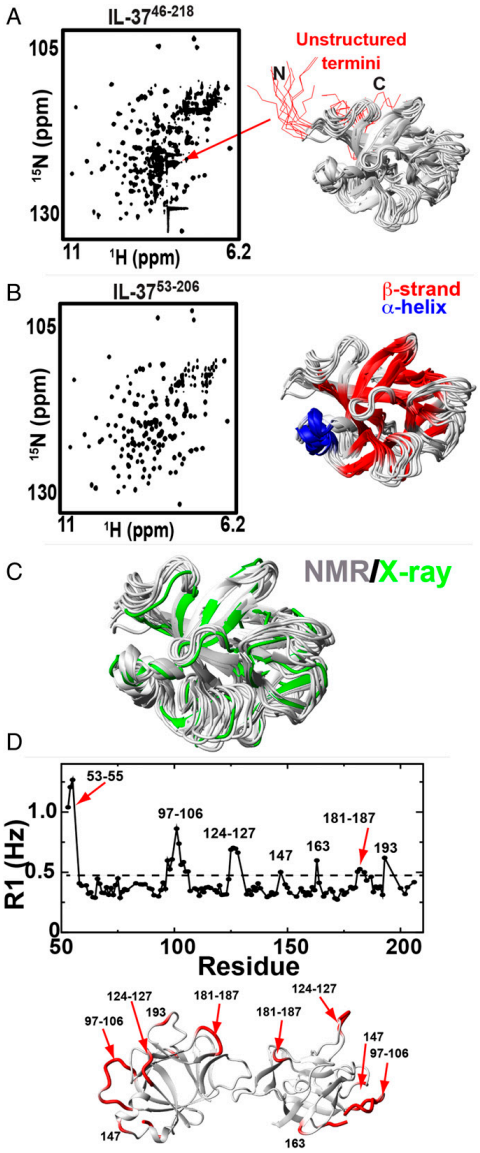

Fig. 2. NMR solution studies of IL-37. (*A*, *Left*) ^{15}N-HSQC spectra of IL-37$^{46–218}$. (*A*, *Right*) Structural ensemble of the 10 highest scoring structures showing residues 46–218 calculated from resolution adapted structural recombination (RASREC) Rosetta with the flexible termini shown (red). (*B*, *Left*) ^{15}N-HSQC spectra of IL-37$^{53–206}$. (*B*, *Right*) Structural ensemble of the same 10 highest scoring structures with a secondary structure of α-helices (blue) and β-strands (red) mapped onto the ensemble calculated using measured chemical shifts and predictions calculated in the chemical shift index (33). Calculated β-strands include residues 59–63, 69–80, 90–97, 109–113, 119–124, 131–137, 155–161, 164–169, 175–179, 189–190, and 198–203, and α-helices include residues 140–145 and 148–151. (*C*) Comparison between this solution ensemble (white) and one subunit within the X-ray crystal structure (green) of residues 53–206 shown. (*D*) R1 relaxation rates of IL-37$^{53–206}$ with the average 0.41 ± 0.16 s^{-1} (dashed line) and residues greater than +0.5 SD mapped onto the X-ray crystal structure dimer (red).

results in a HSQC devoid of this spectral crowding (Fig. 2*B*). Moreover, these termini do not alter the chemical environment of the centrally folded region of IL37$^{53–206}$ as the spectra are completely superimposable that suggests there is no structural change to the IL-37 core upon their removal. The determination of the low-resolution solution structure of the monomer guided by our chemical shift assignments (Biological Magnetic Resonance Bank accession number 27729) and experimental amide NOEs facilitated the low-resolution structure of IL37$^{53–206}$ in the context of its native dimer that indicates the solution structure is identical to that determined within the crystal (Fig. 2*C* and Table 2). Thus, these solution studies provide compelling evidence to suggest that the extended termini of the atypical IL-1 family member are disordered in the context of the free protein.

In addition, to determine that the IL-37 termini are disordered, the dynamic nature of the centrally folded IL-37 core was probed using R1 relaxation rates as sensitive markers for local flexibility (Fig. 2*D*). Overall, the R1 relaxation rates are consistent with the relatively large dimer with an average R1 relaxation rate of 0.41 ± 0.16 s^{-1}. Relatively flexible regions exhibiting R1 relaxation rates can be identified by elevated R1 relaxation rates, shown as, at least, 0.5 SD above the average (Fig. 2*C*, dotted line on the left and shown in red mapped onto our X-ray structure on the right). The entire IL-37 dimer interface is relatively rigid, indicating that the loops at this interface are relatively rigid. Conversely, many loops on the periphery of the IL-37 dimer are disordered that include the relative large loop of residues 96–106 and residues 181–187. Whether these loops are "flexible for function" and comprise the binding epitopes will await future studies aimed at assessing IL-37 interactions with its identified receptors.

IL-37 Mutations at the Dimer Interface Remain Monomeric at Millimolar Concentrations. In general, mutations of protein dimer interfaces may disrupt dimer association and thereby simply shift the equilibrium toward a monomeric form with continued association at higher concentrations, begging the question of whether the engineered IL-37 monomers weakly associate. For example, we have previously shown that CXCL8 mutants that disrupt dimer association nevertheless persist in dimer interactions in solution (20). Specifically, R1 relaxation was used to both probe for local disorder and was simultaneously used as a sensitive reporter of the overall tumbling time that reports on the oligomeric state (i.e., monomer versus dimer). R1 relaxation rates were determined for both D73K and Y85A, which were compared with WT IL-37 (all in the context of IL-37$^{53–206}$).

Using the central core of IL-37 residues 53–206 with point mutations of D73K and Y85A, chemical shift perturbations (CSPs) were induced only to the dimer interface (Fig. 3 *A* and *B*), suggesting that there are little to no changes to the global structure. These monomeric forms exhibit average R1 relaxation rates that are nearly twice as high as the WT dimer with average R1 rates of 0.77 ± 0.10 and 0.79 ± 0.10 s^{-1} for D73K and Y85A, respectively (Fig. 3*B*). Such relatively high R1 relaxation rates are consistent with these mutants retaining their oligomeric states as monomers down to the high concentrations necessary for NMR (near millimolar). Additionally, R1 relaxation rates of the individual mutants indicate that their internal dynamics are remarkably similar to the WT IL-37, indicating

Table 2. Structural statistics for the IL-37 monomer 10 NMR solution structure determined using backbone chemical shifts with RASREC Rosetta

NOE distance restraints	234
Short-range (intraresidue)	22
Sequential	91
Medium-range (|i-j|<=4)	46
Long-range (|i-j|>4)	75
Violations >0.5 Å of hard restraints	23 ± 2
Long-range violations >0.5 Å of hard restraints	5 ± 1
Average long-range violation (Å)	0.59
Average rmsd (over all β-strands)	
Backbone (Å)	1.21 ± 0.3
Heavy atom (Å)	1.76 ± 0.39
Ramachandran plot summary	
Most favored regions (%)	83.1
Allowed regions (%)	15.7
Generously allowed regions (%)	0.2
Disallowed regions (%)	1.1

that these point mutations do not incur substantial global effects on the dynamics and that the dimer interface still remains relatively rigid.

IL-37 Exhibits a Unique Dose-Dependent Profile on Multiple Cell Types. Dose dependencies that monitor the antiinflammatory response of recombinant IL-37 on primary cells elicit a distinct response (Fig. 4), which can be rationalized based on the unique dimerization property of this IL-1 family member. Specifically, increasing doses of IL-37 initially blocks the secretion of proinflammatory cytokines in both mouse and human primary cells induced by either PMA or LPS (Fig. 4 *A* and *B*). IL-37 antiinflammatory activity is observed to reach a maximum response after which increased doses of IL-37 become less effective. As a further corroboration of these results, a similar IL-37 dose–response was also observed using J774A.1 (J1) macrophages as an in vitro model system (Fig. 4 *C* and *D*). Collectively, these data further support a role in IL-37 dimerization in suppressing its antiinflammatory activity.

Mechanistically, these dose dependencies reveal surprising characteristics of IL-37 dimer association that include the role of the IL-37 dimer in blocking IL-37 activity induced by the IL-37 monomer. For example, a theoretical derivation of the IL-37 self-association binding isotherm shows that, although increasing concentrations of IL-37 result in the expected relative increase in IL-37 dimers (*SI Appendix*, Fig. S1*A*), the absolute concentration of IL-37 monomers still increases (*SI Appendix*, Fig. S1*B*). Collectively, these data suggest that the increase in IL-37 dimers inhibit the increase in IL-37 monomer activity, potentially by occluding monomer binding sites. Moreover, IL-37 dimer formation is likely mediated by endogenous molecules. For example, the self-association constant was initially reported as 5 nM (11), and an even weaker self-association constant was recently reported (12). However, the inflection point where IL-37 concentrations becomes inactive varies with different cell types and is well below the reported self-association constants. This modulation of IL-37 oligomerization may be reminiscent of chemokines that interact with a variety of glycosaminoglycans and may therefore be cell-type dependent. Such a possibility was directly assessed by NMR titrations with heparin below, which is a common glycosaminoglycan that does, indeed, engage IL-37 (see Fig. 8).

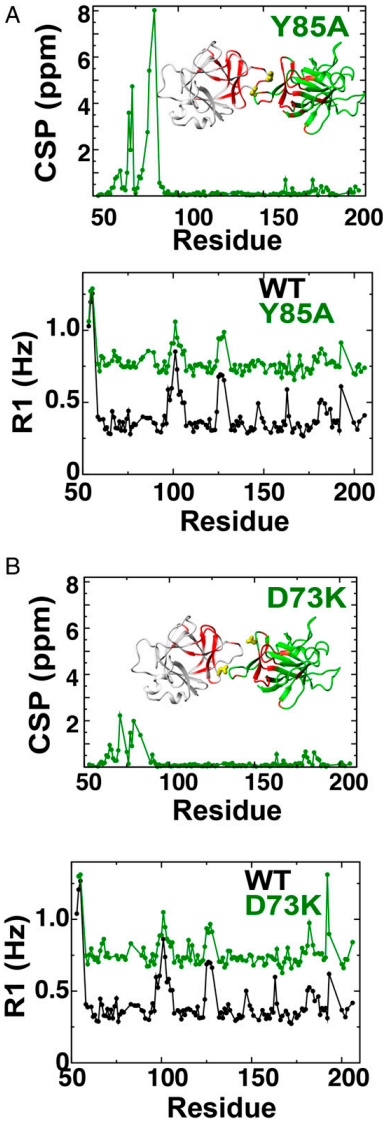

Fig. 3. Solution characterization of IL-37 monomeric mutants. Both amide CSPs and R1 relaxation rates are shown for (*A*) Y85A and (*B*) D73K. CSPs were determined for WTs and mutants in the context of IL-37$^{53–206}$, and R1 relaxation rates are plotted for the WT IL-37$^{53–206}$ together with each mutant form for comparison. Average R1 relaxation rates for Y85A and D73K are 0.79 ± 0.10 and 0.77 ± 0.10 s^{-1}, respectively, consistent with monomer tumbling and compared with the slower average R1 relaxation rate of the WT IL-37$^{53–206}$ dimer with an average of 0.41 ± 0.16 s^{-1}.

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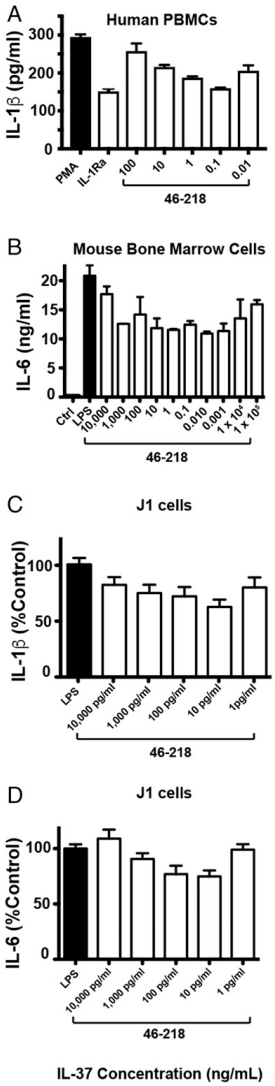

Fig. 4. IL-37 elicits a unique (parabolic) dose dependence observed under multiple conditions. (*A*) Recombinant IL-37 suppression of phorbol 12-myristate 13-acetate (PMA)-induced IL-1β secretion in human peripheral blood mononuclear cells (PBMCs) pretreated with indicated doses of IL-37 2 h before treatment with 25 ng/mL of PMA for 24 h in the presence of 10% FCS. IL-1Ra was also used at 10 μg/mL (*B*) Recombinant IL-37 (IL-$37^{46–218}$) suppression of LPS-induced IL-6 secretion in bone marrow cells of mice pretreated with indicated doses of IL-37 2 h before treatment with 100 ng/mL LPS. Assays were performed in quadruplicate in 96 well plates with 500,000 cells/mL. J1 murine macrophage cells were stimulated with LPS, and the indicated doses of IL-37^{46-218} and supernatants were probed for both (*C*) IL-1β and (*D*) IL-6 as described in Fig. 5.

Either Termini of IL-37 Are Required for Activity and Monomeric Forms of IL-37 Increase the Anti-Inflammatory Activity. The lack of structured termini for IL-$37^{46–218}$ and the suppressive effects of its dimerization facilitate multiple avenues to engineer variants of IL-37 to explore their relative contributions to function. To this end, we sought to directly test the contributions to the activity of the IL-37 termini as well as confirm that IL-37 monomeric mutants enhance IL-37 activity. As J1 cells were shown above to recapitulate the IL-37 activity profiles observed in primary cells, J1 cells were therefore utilized to test the activity of engineered IL-37 forms described herein.

Terminal truncations of the mature WT IL-$37^{46–218}$ were constructed, which include the C-terminally truncated form (IL-$37^{46–206}$), the N-terminally truncated form (IL-$37^{53–218}$), and the central structured form that was subjected above to both X-ray crystallography and NMR studies (IL-$37^{53–206}$). Activities of these terminal truncated forms were compared with WT IL-37 (Fig. 5*A*). Interestingly, the removal of either the N-terminal or the C-terminal residues leads to no measurable change to LPS-induced IL-1β secretion, yet the removal of both termini led to a recombinant form with slightly reduced activity compared with WT IL-37. These data indicate that the removal of the disordered termini of IL-37 do not simply remove regions that are sterically obstructing a single receptor interaction but that there is possibly a more complicated mechanism of engagement. For example, one possibility is that each terminus is critical for specific interactions for specific receptors whereby the other terminus is a negative regulator. Conversely, there may be nonspecific interactions that enhance receptor affinity. Either way, the removal of both termini renders IL-37 unable to bind both receptors.

Both IL-37 monomeric mutations were also tested to confirm that disruption of the WT IL-37 dimer enhances IL-37 activity in the context of the fully mature IL-$37^{46–218}$. Indeed, the activities of both D73K and Y85A are enhanced relative to WT IL-37 (Fig. 5*B*). These data support earlier important findings that indicate the antiinflammatory activity of IL-37 lies in its monomeric form (12), at least, with regard to macrophages that were also extended to primary cells below.

IL-37 Monomers Down-Regulate VCAM-1 Expression in a Dose-Dependent Manner as Opposed to WT IL-37. Consistent with the data described above that utilized cell lines, we studied the dose–response of native IL-$37^{46–218}$ in primary human aortic valve cells (AVICs). As shown in Fig. 6*A*, LPS-induced expression of VACM-1 is reduced from an eightfold increase over the baseline level (normalized to 1.0) to only 1.8-fold by IL-$37^{46–218}$ at 0.05 nM. This low concentration of IL-$37^{46–218}$ is typical of many in vitro studies. However, as shown in Fig. 6*B*, an increase in IL-$37^{46–218}$ to 0.5 nM results in the loss of a significant ability to suppress VCAM-1 expression. In contrast, both mutant monomers in the same assay at 1.0 nM suppressed VCAM-1 expression much better than the WT form. The interpretations of these data are consistent with dimeric forms of WT IL-37 preventing the monomeric IL-37 from access to its receptors. With increasing doses of IL-37 WT and monomeric mutant forms, the higher activity of the IL-37 monomers become increasingly apparent (Fig. 6 *C* and *D*). For example, at 2.5 nM, there was a 50% reduction in VCAM-1 for WT IL-37, but the monomeric mutant D73K reduced expression from eightfold nearly back to the baseline level. Here, the monomeric D73K reduced VCAM-1 from eightfold to 1.8-fold, a 90% decrease. These data support the higher activity of the monomeric IL-37 forms shown above in the cell lines. Thus, using primary cells, we can appreciate the antiinflammatory properties of monomeric IL-37 as the effective form for limiting inflammation. Although monomeric mutants do not exist in nature, the data nevertheless convincingly reveal that the IL-37 monomer is the active form. As discussed below, the ability of low concentrations to be effective in suppressing innate inflammation likely requires a cofactor (s).

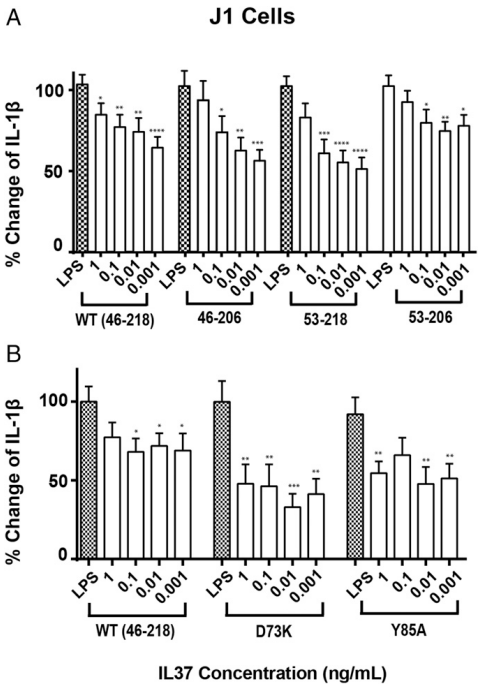

Fig. 5. IL-37-mediated antiinflammatory activity is not increased by terminal truncations but only mutations that disrupt dimer formation. (*A*) J1 murine macrophage cells were pretreated 1 h with the indicated doses of WT IL-37$^{46-218}$ and engineered terminal mutants of IL-37, stimulated with 1 μg/mL LPS for 4 h and, subsequently, treated with 20 μM of nigericin for 1 h before monitoring IL-1β secretion. (*B*) WT IL-37$^{46-218}$ along with both point mutations that specifically disrupt the IL-37 dimer, Y85A, and D73K, was assayed identically to *A*. Assays were performed in triplicate in a 96 well plate with 750,000 cells/mL Cytokine production was quantified by ELISA. All assays are representative of, at least, five independent experiments. Error bars represent the SEM; $*P < 0.05$, $**P < 0.001$, and $***P < 0.0001$, statistical significance was assessed using the unpaired Student's *t* test.

Implications for IL-37 Interactions. Whereas IL-37 has been shown to associate with both IL-18Rα and IL-1R8 (8, 9, 11), studies shown here and previous studies indicate that these interactions are likely more complicated than originally conceived. For example, cononical IL-1 family interactions, such as that of IL-18, first engage a primary receptor followed by the association with a second receptor as is the case for ternary complexes of IL-1/IL-1R/IL-1R3 (21) and IL-18/IL-18Rα/IL-18Rβ (22). Following such a canonical binding mechanism, a straightforward model would suggest that IL-37 first interacts with one receptor (IL-18Rα), and that this is followed by ternary complex formation with a second receptor (IL-1R8) as we have modeled here using the recently determined IL-18 ternary complex (Fig. 7*A*). The evidence for such a model is based on fluorescent studies that identified an IL-37/IL-18Rα/IL-1R8 complex in primary cells and cell lines (9). The elegance of such a model seems appealing as the IL-37 dimer must dissociate in order for the IL-37 monomer to interact with IL-18Rα, which is consistent with our data (Fig. 4),

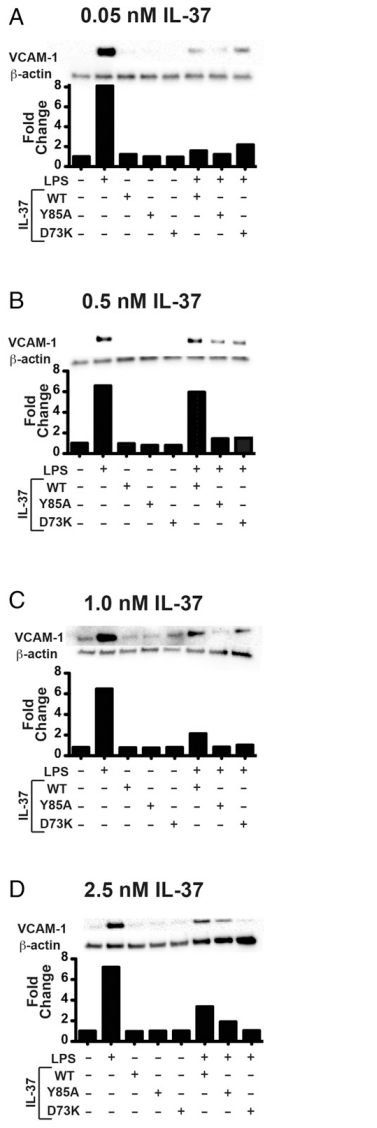

Fig. 6. Suppression of LPS-induced VCAM-1 by high concentrations of IL-37 monomers in human AVICs. (*A*) Normal human AVICs were treated with 0.05 nM recombinant WT IL-37$^{46-218}$, Y85A, and D73K or untreated before stimulation with LPS and then subsequently probed for VCAM-1. Identical assays were performed with (*B*) 0.05 nM, (*C*) 1.0 nM, and (*D*) 2.5 nM recombinant IL-37 proteins.

and the antiinflammatory activity of IL-37 would also inhibit the proinflammatory IL-18 activity. Moreover, superposition of the NMR solution structure ensemble of the monomer in this model suggests that the flexible N terminus would need to reorient to avoid steric clashes with IL-18Rα (Fig. 7*B*). This too may be consistent with the role of one of these termini in dictating IL-37 interactions and the role of the other that may negatively regulate binding (in Fig. 5).

There are important problems with this model that should be addressed in the future. First, the relatively weak affinity of IL-37 to IL-18Rα previously determined as ∼130 nM (11) is inconsistent with the picomolar activity of IL-37 observed here and elsewhere, which may suggest that there are missing cofactors of this interaction. Second, the previously determined 5 nM affinity for IL-37 dimerization would preclude the weaker binding of IL-18Rα in the first place, which further suggests the existence of cofactors. Thus, although the presented canonical mode of IL-37 binding to IL-18Rα and IL-1R8 is consistent with signal transduction in the IL-1 family, there are likely missing cofactors that contribute to the molecular details of IL-37 signaling in full. Such a cofactor (s) may also explain how IL-1R8 functions, which contains only one Ig-like domain compared with, at least, two Ig-like domains for receptors within the canonical interactions.

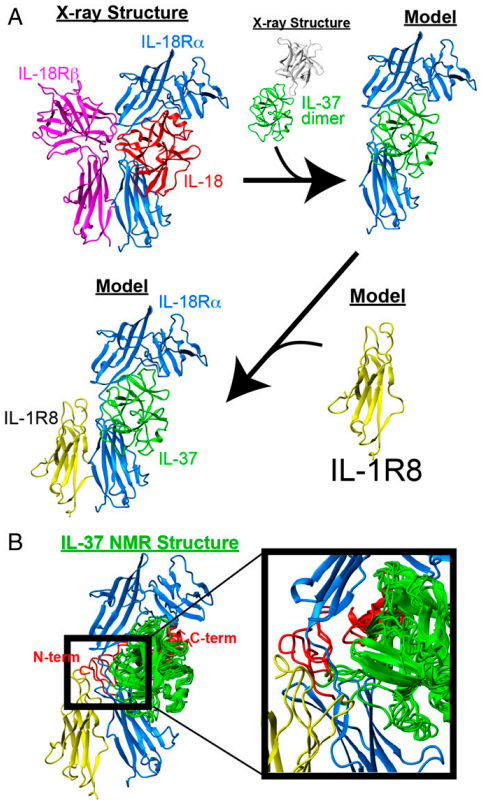

Fig. 7. Potential model of IL-37 interactions. (*A*) Utilizing the IL-18 (red) ternary complex with IL-18Rα (blue), IL-18Rβ (magenta) as a template (PDB accession 3WO4), and an IL-37 monomer (green) from the X-ray crystal structure determined here (green and white) is superimposed onto IL-18 with IL-18Rα and the homology model of IL-1R8 superimposed onto IL-18Rα. Note, the IL-37 dimer is shown in the same orientation of the IL-37/IL-18Ra complex to illustrate the occlusion of the interacting surface within the IL-37 dimer. (*B*) NMR-derived solution ensembles are superimposed onto this model with the mature IL-37 extended termini of residues 46–52 and 207–218 shown (red). A blowup of the N-terminal region of IL-37$^{46-218}$ illustrates potential clashes with the receptor complex, suggesting that this region may need to reorient for interactions.

Finally, to address the findings that IL-37 activity is diminished well below its in vitro determined self-association affinities (11, 12), we hypothesized that glycosaminoglycan interactions are likely to occur and thereby serve to modulate IL-37 interactions. Thus, we sought to directly address whether IL-37 interacts with such endogenous molecules. To test such a scenario, we utilized heparin as a representative of common glycosaminoglycans, which has been shown to modify the activity of several chemokines by stabilizing an oligomeric state (23–25). Indeed, heparin addition to both the WT IL-37$^{53-206}$ dimer (Fig. 8*A*) and the monomeric mutant of Y85A (Fig. 8*B*) induced severe line broadening at stoichiometric concentrations. Although such line broadening may be induced by either oligomerization or binding to multiple interaction sites, the fact that specific CSPs can be observed while simultaneously being diminished in intensities indicates a preferred interaction site with sampling of larger oligomeric species. This simultaneous shifts with concomitant diminishment in intensity is exemplified using Y85A (Fig. 8*C*). A plot of these CSPs reveals a likely heparin binding surface within this monomer (Fig. 8*D*), which is largely distinct from any interaction surface within the modeled ternary complex of IL-37/IL-18Rα/IL-1R8 (Fig. 8*E*). As glycosaminoglycans are present within multiple surface exposed glycoproteins and are abundant within the extracellular matrix (26), these NMR studies identify IL-37 as a potential glycosaminoglycan binding protein. Such interactions may stabilize IL-37 oligomerization by either shifting the monomer/dimer equilibrium toward the dimer or promoting higher order oligomers of the dimer (Fig. 8*D*). Additionally, glycosaminoglycan could compete for an additional cofactor interaction (Fig. 8*E*). In either event, glycosaminoglycan may downregulate IL-37 activity in accord with its inactivation at concentrations well below the self-association affinity.

Conclusion

Revealing the underlying molecular mechanism of IL-37 will identify promising pathways that may be exploited for controlling inflammation. The biophysical, biochemical, and biological studies here confirm that elevated IL-37 concentrations result in a dampening of its antiinflammatory activities whereby dimerization occludes the activity of the IL-37 monomer. Point mutations that specifically disrupt the IL-37 dimer remain stable monomers in vitro and provide greater suppression of inflammatory cytokines compared with native IL-37 on multiple cell types. Athough NMR solution studies have specifically shown that, in the absence of any other interaction, the IL-37 termini are unstructured, the presence of one extended terminus is necessary for activity. Such findings may be reconciled by the existence of multiple cell surface interactions and/or multiple mediators of IL-37 activity, which would also explain how IL-37 is active at picomolar concentrations despite its relatively weak interaction with IL-18Rα.

Materials and Methods

Protein Expression and Purification. Recombinant IL-37 constructs with an N-terminal 6xHis tag and thrombin cleavage site were cloned into pET21 for subsequent expression in BL21/DE3 cells at room temperature. The soluble protein was purified using Ni affinity and subjected to sulphopropyl (SP) ion

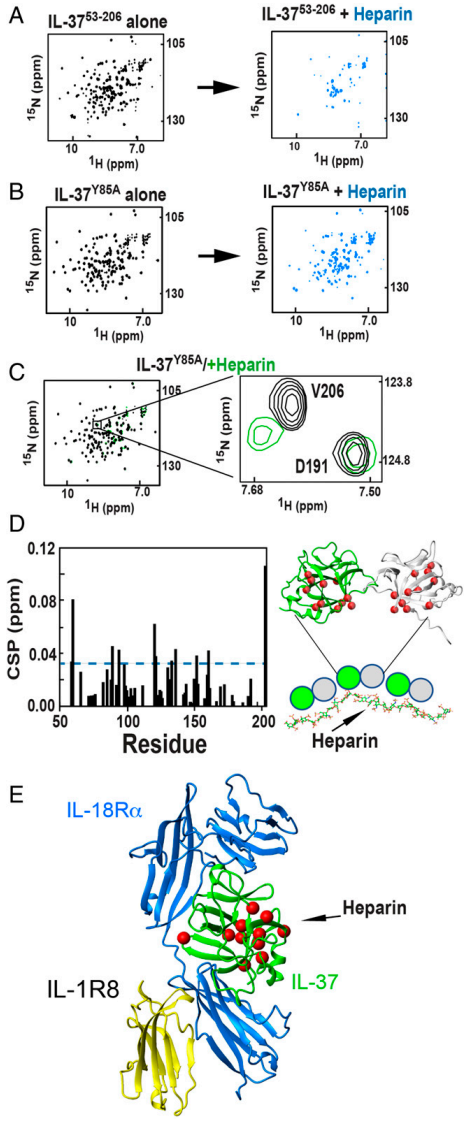

Fig. 8. IL-37 interacts with heparin. Heparin induces severe line broadening in IL-37 ^{15}N-HSQC spectra as shown in a comparison of free protein and equimolar concentrations of heparin for the (*A*) IL-37$^{53–206}$ dimer and (*B*) mutant Y85A. (*C*) ^{15}N-HSQC spectra Y85A (300 μM) with substoichiometric heparin (50 μM), highlighting amide chemical shifts. (*D*) IL-37 residues that exhibit large CSPs in the presence of heparin are largely confined to one surface. (*Left*) Amide CSPs per residue induced by heparin binding within Y85A with substoichiometric heparin added. (*Right*) CSPs greater than 1.0 SD above the average (0.033 ppm) are mapped onto the structural model of the IL-37 dimer (red spheres) that illustrate how either a monomer or a dimer may engage heparin and a representative model of IL-37 interacting with a heparin chain. (*E*) Residues are mapped (red spheres) onto the ternary complex model of IL-37/IL-18Rα/IL-1R8. Mapped residues that exhibit large CSPs upon substoichiometric addition of heparin are K59, F60, I90, A95, C122, D123, L135, E138, F154, S163, and V206.

exchange (50 mM Tris, pH 7.2, 100 mM NaCl, 1 mM EDTA, and 1 mM DTT) with bound IL-37 eluted with high salt (2 M NaCl). After removal of the 6xHis tag with thrombin, the protein was subjected to Superdex-75 size exclusion using the final NMR buffer (50 mM phosphate, 150 mM NaCl, and 1 mM DTT), crystallization buffer (20 mM Trizma, pH 7, 50 mM NaCl, and 1 mM DTT), or biological buffer (PBS). For ^{2}H^{15}N^{13}C-labeled NMR samples, protein was grown at 37 °C and refolded as previously published to facilitate amide exchange (27, 28).

X-Ray Crystallography and NMR Relaxation. Recombinant IL-37$^{53–206}$ was used for all structural studies unless otherwise stated. For NMR assignments, standard BioPack sequences were collected on a ^{2}H^{15}N^{13}C-labeled protein acquired on a 600 MHz Varian spectrometer along with ^{15}N- and ^{13}C-nuclear Overhauser effect spectroscopy (NOESY) spectra acquired on a 900 MHz Varian spectrometer and R1 relaxation data were collected at 900 MHz. Samples were ∼500 μM. Heparin sodium salt from porcine intestinal mucosa (average molecular weight 11–12.5 kDa; Sigma-Aldrich) was added as indicated. Data were processed using nmrPipe software (29) and analyzed using CCPNmr software (30). Chemical shifts and NOEs for the truncated IL-37 residues 53–206 were used with RASREC Rosetta to calculate solution ensembles of the IL-37 monomer (31) with distance restraints all set to 6 Å and IL-37 residues 1–218 used for all solution calculations. For crystallography, conditions were identified within JCSG Core I (Qiagen) condition 92 (0.1 M citric acid pH 2.5, 20% MPD), and crystals were collected at the Advanced Light Source beamline 8.2.1. For computational models, the ectodomain of IL-1R8 residues 1–118 was calculated with the Rosetta structural prediction, and all superpositions were calculated using Coot Software (32).

Cell Lines and in Vitro Assays. The murine macrophage cell line J1 was purchased from American Type Culture Collection and cultured according to the distributor's instructions in DMEM (Corning) supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C and 5% CO_2. For in vitro assays, 75,000 cells per well were plated overnight or 1 h before pretreatment in a flat bottom 96 well plate. J1 cells were stimulated for 4 h with 1 μg/mL LPS; *Escherichia coli* 055:B5; Sigma-Aldrich. For inflammasome activation, 20 μM nigericin sodium salt dissolved in 100% ethanol (InvivoGen) was added for another 1 h. IL-1β and IL-6 concentrations were measured in the cell supernatants by specific ELISA (DuoSet, R&D Systems). Manufacturer's instructions were strictly followed.

Isolation and Culture of Human AVICs. All studies were approved by the Institutional Review Board of University of Colorado (COMIRB; Protocol 08-0280) and performed in accordance with the Declaration of Helsinki. All aortic valve donors gave their written informed consent before their inclusion in this study. The normal tricuspid aortic valves were collected from cardiomyopathy heart-transplant patients at the University of Colorado Hospital.

Aortic valve leaflets were excised, washed in PBS, and then incubated with collagenase solution (type I, 1.0 mg/mL) in hybridization ovens at 37 °C for 30 min to separate endothelial cells. Leaflets were further digested with a fresh solution of 1 mg/mL collagenase solution for 4–6 h at 37 °C to isolate human AVICs. After vortexing and aspirating repeatedly to break up the tissue mass, human AVIC suspensions were centrifuged at 1,000 rpm for 10 min. Pellets were resuspended and cultured in M199 growth medium (Lonza), supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich) in an incubator with 5% CO_2 at 37 °C. The medium was replaced every 3 d throughout the growth, differentiation, and experimental periods. Human AVICs were passaged three to six times and used at 80–90% confluence for all experiments. The AVICs were stimulated with LPS (200 ng/mL) for 24 h. In the experiments, the AVICs were pretreated with recombinant IL-37$^{46–218}$, the single point mutation of D73K, or Y85A for 2 h before the addition of LPS. After 24 h, the cultures were processed for Western blotting with antihuman VCAM-1 antibodies.

Statistical Analysis. Statistical significance was calculated using the two-tailed Student's *t* test with GraphPad Prism 5.0. *P* values < 0.05 were considered to be significant with $*P < 0.05$, $**P < 0.001$, and $***P < 0.0001$.

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