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Immuntherapie des Pankreaskarzinoms: Mechanismen und Strategien zur Durchbrechung tumorinduzierter Immunsuppression

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Inhaltsverzeichnis

1. Einleitung

1.1 Grundzüge des Immunsystems

1.1.1 Das Immunsystem

Das Immunsystem ist das Schutzsystem höherer Lebewesen, welches sicherstellt, dass Pathogene, wie zum Beispiel Mikroben, aber auch fehlgeleitete, körpereigene Zellen erkannt und attackiert werden. Um dieser Aufgabe gerecht zu werden, besteht das Immunsystem aus einem hochkomplexen Netzwerk verschiedener Erkennungs- und Abwehrsysteme, welches sowohl zelluläre wie auch nicht-zelluläre Anteile vereint, schnell reagieren sowie langfristig Immunität sichern kann. Grundsätzlich ist es möglich, ein angeborenes von einem erworbenen, auch adaptiv genannten, Immunsystem zu unterscheiden. Zytokine werden von beiden Systemen als Botenstoffe genutzt. Sie lassen sich also weder dem einen noch dem anderen System exklusiv zuordnen.

1.1.1.1 Das angeborene Immunsystem

Das angeborene Immunsystem ist der evolutionär ältere Teil des Immunsystems und bildet die erste, in der Regel schnelle Abwehr. Hierzu werden sowohl die natürlichen Barrieren des Körpers wie auch bestimmte Zelltypen des Immunsystems gezählt. Besonders zu erwähnen sind die antigenpräsentierenden Zellen (APC) Monozyten/ Makrophagen sowie dendritische Zellen (DC), die nebst eigener Effektorfunktion vor allem Pathogenfragmente aufnehmen und den Zellen des adaptiven Immunsystems präsentieren und somit die entscheidende Verbindung beider Systeme darstellen. Eine Schlüsselrolle nehmen die DC ein, da sie befähigt sind, exogene Antigene nach zellulärer Aufnahme zu prozessieren und auf *major histocompatibility complex* I (MHC-I) zu präsentieren, was der Aktivierung von $CD8^+$ T-Zellen dient. Natürliche Killerzellen (NK-Zellen) wiederum vermögen infizierte, entartete und mittels Antikörper markierte Zellen zu erkennen und zu attackieren.

Eine besondere, heterogene Zellpopulation myeloiden Ursprungs des angeborenen Immunsystems, insbesondere bei Individuen mit Tumoren, sind die *myeloid derived suppressor cells* (MDSC), die verschiedene immunsuppressive Effekte, insbesondere in unmittelbarer Tumorumgebung, vermitteln (Dumitru et al. 2012).

1.1.1.2 Das adaptive Immunsystem

Die adaptive Immunantwort, die langsamer, aber nachhaltiger reagiert, basiert auf den Zellpopulationen der B- und T-Zellen. B-Zellen vermögen, auf einen entsprechenden Reiz hin, Immunglobuline zu produzieren und sich im Rahmen einer akuten Immunreaktion zu Plasma- und langfristig zu Gedächtniszellen zu entwickeln.

T-Lymphozyten bestehen aus mehreren Subpopulationen. $CD4^+$ T-Helferzellen werden durch APC via *major histocompatibility complex* II (MHC-II) Kontakt aktiviert und tragen zur Koordinierung der Immunantwort bei. Sie können in Th1 sowie Th2 $CD4^+$ Zellen unterschieden werden. Im Falle einer Th1 Antwort liegt der Schwerpunkt auf einer zellulären, $CD8^+$ T-Lymphozyten (siehe unten) sowie Makrophagen aktivierenden zytotoxischen Immunantwort inklusive Bildung opsonierender IgG Antikörper. Eine Th2 Antwort hingegen fördert die humorale Immunität (hauptsächlich Bildung von IgM, IgA, IgE). Maßgeblich für eine Entwicklung in die eine oder andere Richtung ist die Zusammensetzung der zum Zeitpunkt der Aktivierung der $CD4^+$ T-Zelle im Milieu vorhandenen Zytokine.

Eine weitere Differenzierung von $CD4^+$ T-Zellen sind so genannte Th3-Zellen, die im Bereich der oralen Toleranz eine entscheidende Rolle spielen. Th17-Zellen wiederum regen im Rahmen von Entzündungen lokal epitheliale und stromale Zellen zur Produktion von Chemokinen an, was wiederum neutrophile Granulozyten anlockt.
$CD4^+CD25^+FoxP3^+$ Zellen werden als regulatorische T-Zellen beizeichnet (Treg) und modulieren die Immunreaktion, indem sie T-Zellaktivität direkt und auch indirekt durch Inhibierung von DC unterdrücken und somit überschießende Immunprozesse sowie Autoimmunität verhindern. Sie inhibieren jedoch auch Effektor T-Zell Aktivität gegen Tumorantigene, wandern ins Tumorstroma ein und sind somit prominent an tumorinduzierter Immunsuppression beteiligt (Bluestone 2005, Betts 2006, Colombo 2007). Dies erklärt wahrscheinlich, dass eine hohe Dichte an Treg im Tumorstroma mit einer schlechten Prognose korreliert (Curiel et al. 2004, Hiraoka et al. 2006, Fu et al. 2007).

Letztlich existiert die Gruppe der $CD8^+$ zytotoxischen T-(Killer) Zellen (CTL), welche ebenfalls durch APC aktiviert werden. In diesem Fall geschieht dies jedoch durch Präsentation von Pathogenfragmenten via MHC-I. Den CTL wird somit ermöglicht, Zellen, welche spezifische Peptide über MHC-I Moleküle auf ihrer Oberfläche präsentieren, zu erkennen und zu attackieren. Tumor-infiltrierende CTL konnten bei

verschiedenen Tumorentitäten als positiver prognostischer Faktor identifiziert werden (Fukunaga et al. 2004).

1.1.1 Erkennung von Gefahrensignalen

Das Immunsystem vermag „Fremd“ von „Selbst“ zu unterscheiden und entsprechend darauf zu reagieren (Chaplin et al. 2010). Wie bereits 2002 von Matzinger postuliert, existieren jedoch zusätzlich allgemeine Gefahrensignale, sowohl exo- wie endogenen Ursprungs, die durch eukaryote Zellen erkannt werden und auf die der Organismus mit einer entsprechenden Immunantwort reagieren kann. Diese invarianten Strukturen werden unterschieden in *pathogen-associated molecular patterns* (PAMP), *microorganism-associated molecular patterns* (MAMP) und *danger-associated molecular patterns* (DAMP). Detektiert werden sie von unterschiedlichen PRR. Hierzu gehören unter anderem die membranständigen Lektinrezeptoren (Banchereau et al. 2000), Scavengerrezeptoren (Peiser 2002), Toll-*like* Rezeptoren (TLR) (Takeda et al. 2005), sowie die zyotosolischen Helikasen *retinoic acid-inducible gene I* (RIG-I) (Yonoyama et al. 2005, Kato et al. 2005), *melanoma differentiation gene* 5 (MDA-5) (Kang et al. 2002) sowie *laboratory of genetics and physiology* 2 (LGP-2) (Rothenfußer et al. 2005). Im Hinblick auf das Spektrum der hier vorgelegten Arbeiten wird sich im Folgenden auf die detailliere Vorstellung der TLR sowie von RIG-I beschränkt.

1.1.1.1 Toll-*like* Rezeptoren (TLR)

Erstmals 1985 durch Anderson et al. identifiziert kann man bei Säugetieren mittlerweile zwischen dreizehn verschiedenen TLR differenzieren. Diejenigen, deren Funktion bekannt ist, sind entweder auf der Zelloberfläche (TLR1, 2, 4, 5, 6) oder in endosomalen Membranen (TLR3, 7, 8, 9), hauptsächlich von Immunzellen, lokalisiert (Akira et al. 2006, Medzhitov 2007, Beutler et al. 2009). Während die erstgenannten molekulare Muster erkennen, die überwiegend in Bakterien zu finden sind, erkennen die letztgenannten, ihrer Lokalisation entsprechend, in das Zellinnere eingedrungene Virusgenommuster im Rahmen von viralen Infektionen. Im Detail werden Doppelstrang-RNA von TLR3, Einzelstrang-RNA von TLR7, Guanin-reiche Oligonukleotide von TLR8 und Cytosin-Phosphat-Guanin-Oligodesoxynukleotide (CpG-ODN) von TLR9 erkannt (Krieg 2002, Barton et al. 2002, Beutler et al. 2004, Kanzler et al. 2007). Nach Aktivierung kommt es einerseits zur Aktivierung von *mitogen-activated protein kinases* (MAP-Kinasen), andererseits zur Translokation von *interferon regulatory factor*-3 und -7 (IRF-3 und -7) und *nuclear factor κB* (NF-κB) in den Nukleus durch Assoziation von *myeloid differentiation primary response gene* 88 (MyD88) mit *IL-1 receptor-associated kinase* (IRAK). Eine Ausnahme bildet die Aktivierung von TLR3, bei der es zur Bindung an *TIR-domain-containing adapter-inducing interferon-β*

(TRIF) kommt. Das Ergebnis ist eine (verstärkte) Sekretion von proinflammatorischen Zytokinen sowie Typ 1 Interferonen (siehe auch Abbildung 1).

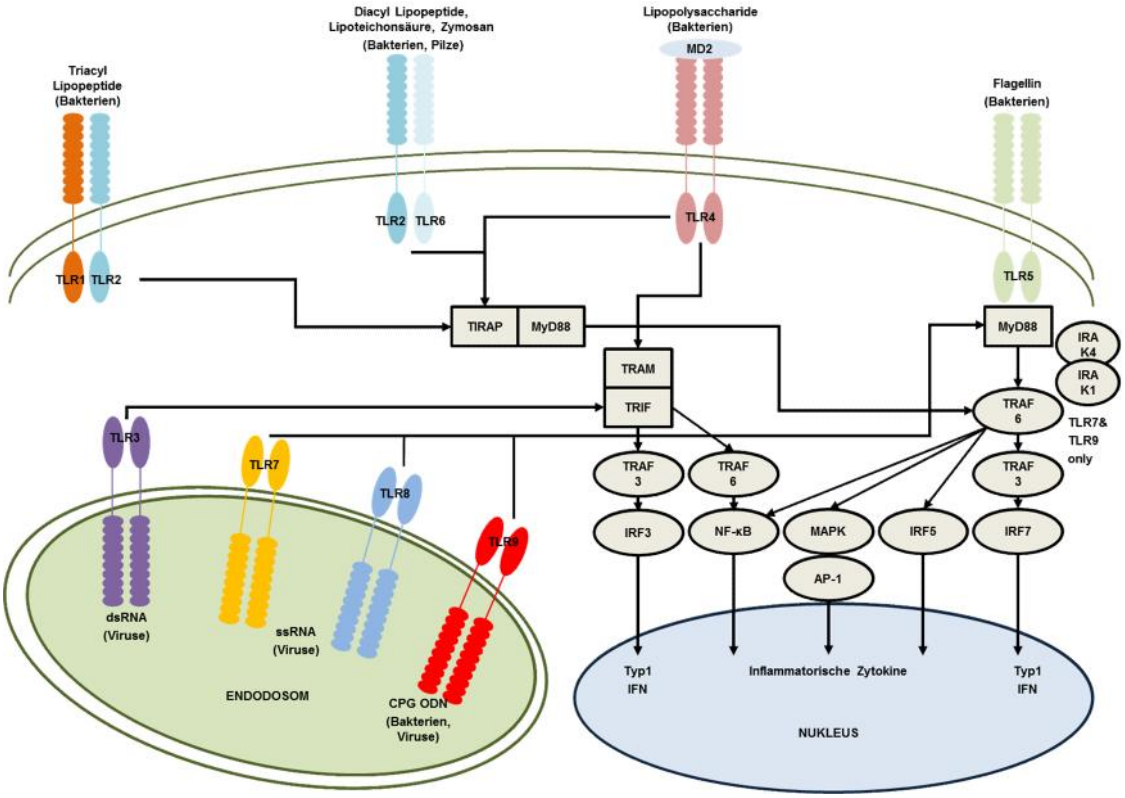

Abbildung 1: TLR Signalwege. Abbildung nach Adams, 2009

Die Rolle von TLR in der Karzinogenese ist erst unvollständig verstanden. Neuere Arbeiten postulieren eine fördernde Rolle von TLR7 am Beispiel des Pankreaskarzinoms (Ochi et al. 2012). Andererseits belegeteine Vielzahl von Arbeiten, dass TLR Liganden, als Therapeutika eingesetzt, effektive antitumorale Effekte vermitteln. Der TLR7/8 Agonist Imiquimod wird vor allem bei dermatologischen Malignomen klinisch eingesetzt. Bacillus Calmette-Guerin (aktiviert TLR2/4) ist eine Standardbehandlung bei Blasenkarzinomen. Weitere TLR aktivierende Wirkstoffe befinden sich in klinischer Erprobung (Adams 2009, Galluzzi et al. 2012).

1.1.2.2 *RIG-I like* Helikasen (RIG-I)

Die RIG-I ähnlichen Helikasen/ATPasen RIG-I, MDA-5 sowie LPG-2 finden sich im Zytosol von Immun-, aber auch den meisten Nicht-Immunzellen. Sie gehören zu den *superfamily-2* (SF2) Helikasen und teilen sich sieben konservierte Motive, welche die Nukleinsäure- und ATP-Bindung vermitteln (Gorbalenya et al. 1988, Hopfner et al. 2007). Während MDA-5 Doppelstrang-RNA detektiert und artifiziell durch synthetische *polyinosinic:polycytidylic acid* (poly[I:C]) aktiviert werden kann (Kang et al. 2002, Gitlin et al. 2006, Kato et al. 2006), erkennt RIG-I hautsächlich eine Triphosphatgruppe, die typischerweise von viralen Polymerasen im Zytosol der Zelle im Rahmen von Replikationsabläufen am 5‘-Ende von Doppelstrang-RNA Molekülen generiert wird

(Kato et al. 2005, Hornung et al. 2006, Saito et al. 2008). Zusätzlich bedarf es eines kurzen basenpaarigen Abschnitts der RNA, welcher bei Einzelstrang-RNA durch eine *loop*-Struktur ermöglicht wird (Schmidt et al. 2009). Erwähnenswert ist, dass die Triphosphatgruppe am 5‘-Ende eines RNA-Strangs auch regelmäßig physiologisch im Nukleus von Zellen generiert wird, jedoch aufgrund von Spleißungsvorgängen, 5‘-*capping* und weiteren Modifikationen normalerweise nicht in direkten Kontakt mit RIG-I im Zytosol kommt (Pichlmair et al. 2006, Yoneyama et al. 2009). 5‘-Triphosphat RNA kann mittels *in-vitro*-Transkription synthetisch hergestellt und mittels Transfektion in die Zielzellen eingeschleust werden, wodurch sich neue Therapieoptionen ergeben (Kato et al. 2005, Hornung et al. 2006). Die Rolle von LGP-2 ist bisher nicht vollständig verstanden, es wird jedoch eine eher regulatorische Funktion angenommen (Rothenfußer et al. 2005, Pippig et al. 2009).

1.2.2.3 Die RIG-I Signalkaskade mit Typ 1 Interferon Aktivierung

Nach Erkennung der Triphosphatgruppe initiiert RIG-I mit Hilfe des Adapterproteins *interferon promoter stimulator* 1 (IPS-1) (auch als CARDIF, MAVS oder VISA bekannt) eine Signalkaskade, welche durch IRF-3, IRF-7 sowie NF-κB reguliert wird (Kawai et al. 2005, Meylan et al. 2005, Xu et al. 2005, Sun et al. 2006). Diese Signalkaskade führt schließlich, ähnlich der TLR Signalkaskade, durch Wanderung von phosphorylierten IRF-3 und IRF-7 Homo- sowie Heterodimeren in den Nukleus, zur Transkriptionsaktivierung von Typ 1 Interferonen (IFN-α, IFN-β) und proinflammatorischen Zytokinen (Kato et al. 2006, Hornung et al. 2006).

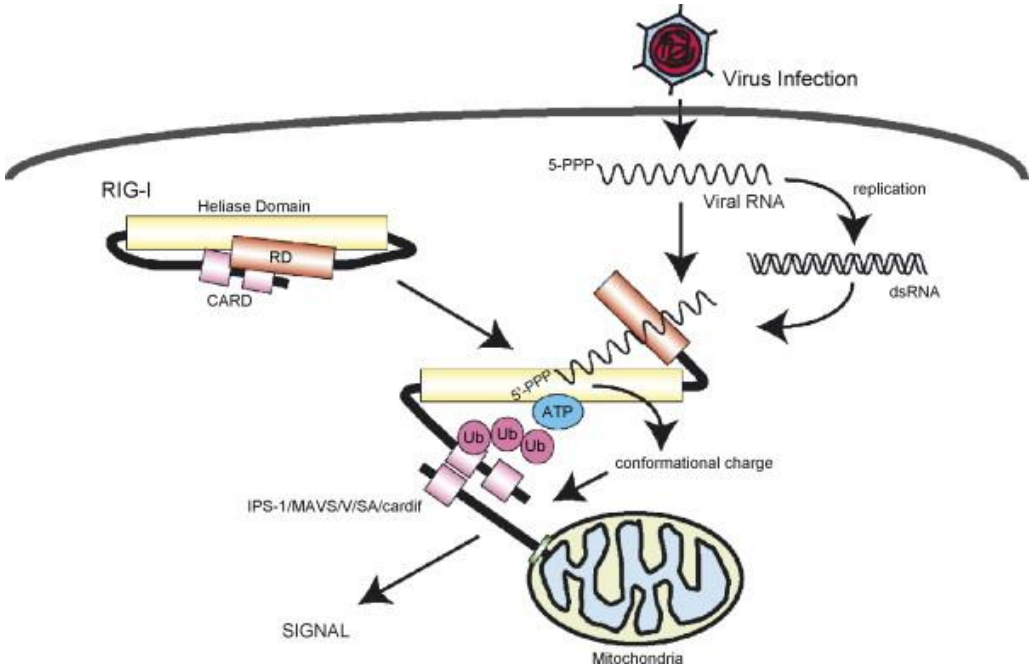

Abbildung 2: RIG-I Signalweg nach ppp-RNA Stimulation. Abbildung nach Yoneyama, 2007

Typ 1 Interferone sind Zytokine, die vor allem von Leukozyten, Monozyten und Fibroblasten produziert werden, jedoch in geringerem Maße auch von anderen Zellpopulationen, inklusive Tumorzellen, gebildet werden können. Sie greifen direkt in die intrazelluläre Virusreplikation ein und unterbinden diese, hauptsächlich vermittelt über den Januskinasen-*signal transducer and activator of transcription* (JAK-STAT) Signalweg (Platanias 2005). Nach Bindung an Interferon-Rezeptoren auf den Ursprungszellen sowie umgebenden Zellen werden, vermittelt über den erwähnten JAK-STAT Signalweg, MHC-I Moleküle verstärkt auf der Zelloberfläche exprimiert, um Attacken durch zytotoxische $CD8^+$ T-Zellen zu erleichtern. Aktivierte T-Zellen werden am Leben gehalten. DC, Makrophagen und NK-Zellen werden aktiviert, sofern sie nicht bereits selbst virusinfiziert sind und somit bereits autokrin aktiviert wurden. B-Zellen werden zur Bildung von Antikörpern animiert. Des Weiteren werden verschiedene IFN-abhängige Botenstoffe der angeborenen Immunität, wie das Chemokin CXCL10, das Interleukin 2 (IL-2) oder der *tumor necrosis factor alpha* (TNFα), hochreguliert. Im Zusammenspiel mit p53 kommt es zu Apoptose der infizierten Zelle (Takaoka et al. 2003, Dunn et al. 2005, Platanias et al. 2005, Pestka 2007, Fensterl et al. 2009).

Zusätzlich kommt es nach Aktivierung der RIG-I-like Helikasen Interferon-unabhängig zu Apoptose auf dem intrinsischen beziehungsweise mitochondrialen Pfad durch Aktivierung der *BH3-only* Proteine Puma, Noxa und in geringerem Maße Bim und Bik (Besch et al. 2009). Interessanterweise scheinen maligne Zellen für diesen Apoptosemechanismus besonders anfällig zu sein. Der Grund hierfür liegt wahrscheinlich im Schutz nicht-maligner Zellen durch BCL-xl, das in Tumorzellen im Rahmen der so genannten *synthetic lethality* und *oncogene addiction* nicht vorhanden beziehungsweise nicht funktionstüchtig zu sein scheint, da durch Genalterationen im Prozess der Karzinogenese eine erhöhte Vulnerabilität entstehen kann (Hartwell et al. 1997, Evan 2006).

1.2 Tumorimmuntherapie

1.2.1 Allgemeiner Status Quo

In den vergangenen Jahren ist eine Vielzahl an Ansätzen verfolgt worden, um, jenseits von Chirurgie, Strahlen- und Chemotherapie, auch die Immunantwort des Organismus für die Tumortherapie zu nutzen. Mittlerweile hat sich die Immuntherapie als viertes Standbein der Onkologie etabliert. Das therapeutische Spektrum erstreckt sich vom Einsatz onkolytischer Viren, Aktivierung von PRR oder der adoptiven T-Zell Therapie über den Einsatz extrakorporaler DC-Aktivierung bis hin zur großen Gruppe

immunmodulierender Antikörper, um nur einige der Strategien zu benennen (Krieg 2007, 2008, Vollmer et al. 2009, Bauer et al. 2011, Wu et al. 2012). Ein zentrales Problem der Immuntherapie besteht darin, dass viele Malignome immunsuppressive Eigenschaften aufweisen, was die Effektivität der genannten Strategien limitiert (Armstrong et al. 2001, Franks et al. 2012, Hong et al. 2012). Gerade das Pankreaskarzinom wartet mit einer Vielzahl immunsuppressiver Eigenschaften auf, die eine Barriere für eine effektive Immuntherapie darstellen.

1.2.2 Immuntherapie des Pankreaskarzinoms

In den letzten Jahren wurden auch in der Behandlung des Pankreaskarzinoms eine große Bandbreite therapeutischer Ansätze inklusive Vakzinen, monoklonalen Antikörpern sowie T-Zell und DC Therapien mit unterschiedlichem jedoch grundsätzlich nicht durchschlagendem Erfolg untersucht (Bauer et al. 2011, Dodson et al. 2011, Michl et al. 2013). Aktuell werden mehrere experimentell vielversprechende Immuntherapeutika klinisch evaluiert. Hierzu gehören die cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) Antikörper Ipilimumab und Tremelimumab, welche die Herunterregulation aktivierter T-Zellen verhindern können, das Fusionsprotein L19-IL2, welches durch Bindung an die tumorspezifische *extradomain* B (ED-B) extrem hohe IL-2 Spiegel lokal im Tumorgewebe induziert, wie auch ein CD40 Agonist, der die T-Zell Aktivierung sowie die Aktivierung myeloider Zellen im Tumorstroma unterstützt (Wagner et al. 2008, Hodi et al. 2010, Beatty et al. 2011). Bisher konnte jedoch kein immunologischer Ansatz als Standardtherapie etabliert werden.

1.3 Das Pankreaskarzinom

1.3.1 Epidemiologie, Diagnose und Therapie

Das Pankreaskarzinom ist die vierthäufigste Todesursache durch Krebserkrankungen weltweit, obwohl die Inzidenz nur bei etwa 15 pro 100.000 Einwohnern und damit verhältnismäßig niedrig liegt. Die zusammengefasste Fünf-Jahres-Überlebensrate liegt bei unter fünf Prozent. Eine Heilung ist nur durch eine, in durchschnittlich weniger als 15 Prozent der Fälle gelingende, chirurgische R0-Resektion möglich. Einzig bei Diagnose im UICC Stadium I-II (lokal begrenzt, keine Metastasen) erscheint die Fünf-Jahre-Überlebensrate mit 40 Prozent vielversprechender. Eine frühzeitige Diagnose ist jedoch nur selten möglich, da die Erkrankung oft lange asymptomatisch bleibt (Jemal et al. 2008). Der chemotherapeutische Goldstandard in der palliativen Situation, bei der eine Operation nicht mehr möglich beziehungsweise sinnvoll ist, sowie adjuvant nach erfolgreicher R0-Resektion, ist das Zytostatikum Gemcitabin. Das Pankreaskarzinom

ist jedoch weitgehend chemo- und strahlentherapieresistent, so dass große Fortschritte bezüglich der Heilungsraten bisher kaum erzielt werden konnten (Burris et al. 1997, Vulfovich et al. 2008). Die Polychemotherapie nach dem FOLFIRINOX-Schema hat sich beim metastasierten Pankreaskarzinom als effektiver erwiesen, kann jedoch aufgrund seiner hohen Toxizität nur bei Patienten in sehr gutem Allgemeinzustand eingesetzt werden (Conroy et al. 2011).

1.3.2 Ätiologie, Pathologie und Pathogenese

Es sind eine Reihe von Risikofaktoren inklusive Zigarettenrauchen, Alkoholkonsum, Adipositas, chronischer Pankreatitis sowie zystischer Pankreasneoplasien beschrieben worden. Darüber hinaus bestehen mehrere Tumordispositionssyndrome mit unterschiedlichem Erkrankungsrisiko, wie das Peutz-Jeghers-Syndrom (STK11 Gen), die hereditäre Pankreatitis (PRSS1 Gen) und das familiäre Pankreaskarzinom (Gen unbekannt), um die drei wichtigsten zu nennen.

Unterschieden wird zwischen dem weitaus häufigeren duktalen (circa 90 Prozent) und dem azinären Karzinom (circa 10 Prozent). Lokalisiert sind beide Typen in circa 70 Prozent im Bereich des Pankreaskopfes.

Ausgangspunkt der Tumorprogression ist in 85 bis 95 Prozent der Fälle eine Mutation des Onkogens Kras. In 60 bis 80 Prozent der Fälle kann weiterhin eine Genmutation der Tumorsuppressoren p15 und/ oder p16 sowie in je etwa 50 Prozent der Fälle eine Mutation in den Tumorsuppressorgenen von p53 und DPC4/ Smad4 detektiert werden (Wong 2009). Die Mutation des p53 Gens bedeutet, dass Apoptose über den extrinsischen Pfad nur noch eingeschränkt möglich ist, da hierbei p53 als wichtiger Induktor wirkt (Igney et al. 2002, Haupt et al. 2003). Zusätzlich findet sich in vielen Pankreaskarzinomen eine Mutation des *transforming growth factor-beta* Rezeptors (TGF-βR) mit nachfolgend gestörter Signalkaskade (Massagué et al. 2008). Neuere Erkenntnisse sprechen auch chronischen Entzündungsvorgängen, die zur Entstehung von Tumorstroma beitragen, einen zentralen Anteil an der Kanzerogenese zu (Ochi et al. 2012).

1.3.3 Die Aggressivität und Letalität des Pankreaskarzinoms

Neben der meist späten Diagnosestellung in entsprechend fortgeschrittenem Erkrankungsstadium wurden zwei weitere Hauptgründe bisher als Erklärung der hohen Letalität des Pankreaskarzinoms beschrieben: die desmoplastische Reaktion sowie die geringe Immunogenität mit ausgeprägter tumorinduzierter Immunsuppression.

1.3.3.1 Desmoplastische Reaktion

Im Laufe seines Wachstums schafft sich das Pankreaskarzinom eine bindegewebsreiche, privilegierte Wachstumsumgebung. Im Gegensatz zu anderen Tumorentitäten, die oft als geballter Karzinomzellhaufen mit intensiver Gefäßversorgung imponieren, zeigt sich das Pankreaskarzinom als derbe, schlecht vaskularisierte Masse. Dies führt unter anderem dazu, dass der Abstand zwischen Blutgefäß und Karzinomzelle zu groß für eine effektive Chemotherapie ist und auch Zellen des Immunsystems schlechter das Tumorgewebe infiltrieren können (Wong et al. 2009, Olive et al. 2009, Neesse et al. 2011, Michl et al. 2013).

1.3.3.2 Immunmodulation und tumorinduzierte Immunsuppression

Pankreaskarzinomzellen verfügen über eine nur schwach immunogene Zelloberfläche mit sehr geringer MHC-I Expression (Costello et al. 1999). Es fehlen Adhäsions- und ko-stimulatorische Moleküle, was die Anheftung und Aktivierung von Immunzellen erschwert (Rabinovich et al. 2007). Gerade im Gegensatz zu virusinduzierten Tumoren fehlen Interferone als immunaktivierende Signale. Somit besteht für das Immunsystem möglicherweise kaum eine Chance, längerfristig eine Eliminations- oder zumindest eine Equilibriumsphase im Sinne des Immunüberwachungsmodells aufrechtzuerhalten (Dunn et al. 2002). Entscheidend ist außerdem eine tumorinduzierte Immunsuppression durch verschiedene Mediatoren beziehungsweise die Blockade solcher (Rayman et al. 2000, von Bernstorff et al. 2002, Rabinovich et al. 2007). Es kommt zu einer Rekrutierung von Treg in das Tumorstroma und zu T-Zell Anergie (Sakaguchi 2008, Liyanage et al. 2002, Fukunaga et al. 2004, Thomas et al. 2005, Massagué et al. 2008). Eine zentrale Rolle in all diesen immunsuppressiven Vorgängen spielt das Zytokin *transforming growth factor-beta* (TGF-β), welches im Tumorgewebe überexprimiert wird.

1.4 *Transforming growth factor*-beta (TGF-β)

TGF-β mit seinen Unterklassen TGF-β_1, TGF-β_2 und TGF-β_3 ist ein Zytokin, dass 1983 erstmals aus Kulturüberständen von Tumorzellen isoliert wurde (Assoion et al. 1983, Frolik et al. 1983, Roberts et al. 1983). Es bindet an Serin-Threonin Kinasen und beeinflusst die Transkription unterschiedlichster Gene (Massagué et al. 1996). Unter physiologischen Bedingungen sichert TGF-β die Gewebshomöostase durch Kontrolle der Zellproliferation und des Zellüberlebens, der Zelldifferenzierung sowie der Zelladhäsion. TGF-β wird allgemein als potentester, natürlich auftretender Unterdrücker von Immunfunktion angesehen, unverzichtbar zur Vermeidung von Autoimmunität (Pennison et al. 2007). TGF-β *knockout* Mäuse haben sich als nicht

dauerhaft lebensfähig gezeigt (Tang et al. 1998). Beispielsweise wird orale Toleranz durch TGF-β induzierten Antikörperswitch zu IgA sowie Th3-Zell Aktivierung ermöglicht (Gilbert et al. 2011). T-Zellen, die im Falle von entzündlichen Darmerkrankungen für die überschießende Immunreaktion verantwortlich sind, verfügen über pathologisch hochreguliertes SMAD7, was eine reduzierte Reaktion auf TGF-β vermittelte, inhibitorische Signale bewirkt (Becker et al. 2006). Bei Malignom-induzierter Hypersekretion oder TGF-β Rezeptormutation mit Fehlregulation der anhängigen Signalkaskade kommt es jedoch zu einer das Tumorwachstum fördernden, massiven Immunsuppression (Biswas et al. 2004, Ijichi et al. 2006).

Hohe Spiegel von TGF-β im Blut von Pankreaskarzinompatienten korrelieren mit einer schlechten Prognose (Friess et al. 1993). TGF-β wird eine entscheidende pro-metastatische Rolle zugesprochen (Bhowmick et al. 2004, Pollard et al. 2004, Kallari et al. 2006). Die Präsenz von TGF-β an der invasiv wachsenden Seite eines Tumors wird mit Tumorprogression und Metastasierung assoziiert (Dalal et al. 1993, Padua et al. 2009). Es induziert eine *epithelial-to-mesenchymal transition* (EMT) der Karzinomzellen und erhöht dadurch deren Motilität und damit den Grad der Invasivität der Tumorzellen (Ellenrieder et al. 2001, Bhowmick et al. 2001, 2004, Drabsch et al. 2012). Die Angiogenese wird zudem gefördert (Roberts et al. 1983). TGF-β ist beteiligt an der Induktion, der Rekrutierung und Expansion von MDSC (Li et al. 2012). Darüber hinaus wirkt es auch direkt inhibierend auf Makrophagen, B-Zellen und CTL, reduziert deren Teilungsrate sowie ihre Fähigkeit, fremde Zellen zu erkennen und zu attackieren. Im Detail blockiert es die Bildung und Sekretion von Perforin, Granzym, Fas-Ligand sowie Interferon-γ (IFN-γ), was eine weitestgehende $CD8^+$ T-Zell Anergie zur Folge hat (Fukunaga et al. 2004, Thomas et al. 2005, Massagué et al. 2008). TGF-β verschiebt die Immunantwort des Organismus im Gesamten von einer Th1 dominanten, zytotoxischen zu einer Th2 fokussierten Immunantwort, beziehungsweise induziert auch direkt die Bildung regulatorischer T-Zellen (Chen et al. 2003, Moutsopoulos et al. 2008). Aus den genannten Gründen kann TGF-β als ein zentrales Molekül der tumorinduzierten Immunsuppression bezeichnet werden.

1.4.1 TGF-β als therapeutische Zielstruktur für die Tumorimmuntherapie

Aufgrund der beschriebenen Eigenschaften erscheint TGF-β als sinnvolles Ziel einer Therapie des Pankreaskarzinoms, die auf die Brechung tumorinduzierter Immunsuppression fokussiert ist. Einige Anti-TGF-β Moleküle waren in präklinischen Studien bereits effektiv und ein Teil von ihnen wird aktuell in klinischen Studien bei Patienten mit Melanomen, Glioblastomen, kolorektalen Karzinomen, Nieren-, Brust-

und auch Pankreaskarzinomen untersucht (Schlingensiepen et al. 2006, Gaspar et al. 2007, Schlingensiepen et al. 2009, Takaku et al. 2010, Drabsch et al. 2012). Hierbei werden unterschiedliche Strategien verfolgt, wobei meist versucht wird, TGF-β und seine Wirkung so hoch wie möglich im Signalweg zu neutralisieren. Dies kann entweder durch die Inhibition oder Sequestrierung der TGF-β Protein Liganden, des Proteins selbst oder durch die Blockade der TGF-β Rezeptoren erfolgen. Hierfür werden *small molecules*, *antisense* Oligonukleotide, *small hairpin* RNA oder auch neutralisierende Antikörper verwendet. Teilweise wurden diese Strategien bereits in Kombination mit konventionellen Therapien (Chemotherapie, Radiotherapie) oder Immuntherapien wie dem adoptiven T-Zell Transfer untersucht. Trotz einigem therapeutischen Erfolg bleiben Zweifel bezüglich der systemischen Nebenwirkungen mit der Gefahr von *de novo* Tumoren oder Autoimmunprozessen aufgrund der vielschichtigen Eigenschaften von TGF-β (Drabsch et al. 2012).

1.5 Zusammenfassung/ *Summary*

1.5.1 Zusammenfassung der vorgelegten Publikationen

In den letzten Jahrzehnten konnten große Erfolge in der Tumortherapie gefeiert werden, auch und besonders auf dem Feld der Tumorimmuntherapie. Bei einigen Tumorerkrankungen, allen voran beim Pankreaskarzinom, blieben die therapeutischen Fortschritte jedoch dürftig. Als ein zentraler Grund hierfür wird die tumorinduzierte Immunsuppression angesehen. Daher bedarf es kontinuierlicher Anstrengung, die Mechanismen dieser Immunsuppression weiter zu verstehen und aus den gewonnenen Erkenntnissen Therapieansätze zu entwickeln, die letztlich auch Einzug in den klinischen Alltag halten können.

1.5.1.1 Anz et al. Int J Cancer 2011.

CD103 is a hallmark of tumor-infiltrating regulatory T-cells.

Der erste Teil der Arbeit behandelt die Rolle der $CD103^+$ (auch bekannt als αEβ7) Subpopulation regulatorischer T-Zellen (Treg). Treg spielen eine entscheidende Rolle im Rahmen tumorinduzierter Immunsuppression. Die gegen Treg bereits verwendeten, beziehungsweise theoretisch denkbaren Therapieoptionen, haben den Nachteil, dass es sich entweder um schlecht erreichbare, intrazelluläre Zielstrukturen handelt (FoxP3), die Effektivität mit steigender Tumorlast massiv abnimmt (CD25) oder das Ziel zu unspezifisch ist und es somit zu autoimmunen Nebenwirkungen kommt (CTLA4) (Onizuka et al. 1999, Kapadia et al. 2005, Colombo et al. 2007).

Initial konnten wir die gesteigerte immunsuppressive Potenz $CD103^+$ im Vergleich mit $CD103^-$ regulatorischen T-Zellen bestätigen. Es gelang durch Analysen in vier verschiedenen murinen Tumormodellen (EL4 Lymphom, CT26 Kolonkarzinom, B16 Melanom, Panc02 Pankreaskarzinom) zu zeigen, dass die Population $CD103^+$ Treg spezifisch für tumorinfiltrierende Treg ist, jedoch CD103 nicht für die Retention dieser im Tumorstroma verantwortlich zeichnet. Unsere Ergebnisse lassen weiterhin darauf schließen, dass intratumorales TGF-β entscheidend an der Induktion der $CD103^+$ Subpopulation beteiligt ist, da unter anderem im murinen, orthotopen Panc02 Pankreaskarzinommodell nach systemischer Therapie mit einer siRNA gegen TGF-β die Zahl $CD103^+$ Treg signifikant reduziert werden konnte. Zusammenfassend lässt sich sagen, dass CD103 eine potentielle Zielstruktur für die Therapie tumorinduzierter Immunsuppression darstellt.

1.5.1.2 Jacobs et al. Int J Cancer 2011.

An ISCOM vaccine combined with a TLR9 agonist breaks immune evasion mediated by regulatory T-cells in an orthotopic model of pancreatic carcinoma.

Im zweiten Teil der Arbeit haben wir in einem murinen, orthotopen Pankreaskarzinommodell eine Vakzinierungsstrategie gegen Tumorantigene mittels immunstimulatorischer Komplexe (ISCOM) untersucht. ISCOM-Vakzine bestehen aus Proteinantigenen, die mit käfigartigen Nanostrukturen, die aus Saponin, Phospholipiden und Cholesterin aufgebaut sind, komplexiert werden. ISCOM-Vakzine induzieren eine allgemeine Immunstimulation sowie B- und T-Zell-vermittelte Immunantworten gegen multiple MHC-II und MHC-I Epitope der entsprechenden Proteinantigene. Maßgeblich ist eine Aktivierung von DC, die zur Antigenkreuzpräsentation und nachfolgender T-Zell Aktivierung befähigt werden (Davis et al. 2004, Schnurr et al. 2005, Drane et al. 2007, Schnurr et al. 2009, Duewell et al. 2011).

Für diese Versuche verwendeten wir eine OVA/ISCOM-Vakzine und generierten Panc02 Tumorzellen, die Ovalbumin (OVA) als experimentelles Tumorantigen exprimieren (PancOVA). Der Impfstoff wurde alleine sowie in Kombination mit dem TLR9 Agonisten CpG-ODN 1826 subkutan injiziert. Zudem wurde eine Kombination der Vakzine mit einem gegen das Oberflächenmolekül CD25 gerichteten Antikörper evaluiert, welcher zu einer Depletion von Treg führt.

Die prophylaktische Gabe der OVA/ISCOM Vakzine führte zu einem vollständigen Tumorschutz durch die hocheffektive Induktion OVA-spezifischer CTL. Bei bereits

etablierten Tumoren (therapeutische Vakzinierung) jedoch war die alleinige Gabe der OVA/ISCOM Vakzine nicht ausreichend effektiv. Dies war unter anderem auf eine tumorvermittelte Induktion von Treg zurückzuführen. Entsprechend verbesserte die Gabe des CD25 Antikörpers das Therapieergebnis signifikant. Die Kombination der Vakzine mit dem TLR9 Agonisten CpG ODN 1826 führte zu einer Th1-dominanten Immunantwort mit Aktivierung von Immunzellen des angeborenen sowie des adaptiven Immunsystems. Daraus resultierte eine massive Expansion von Antigen-spezifischen $CD8^+$ CTL. Therapeutisch konnte eine signifikante Lebensverlängerung inklusive kompletter Tumorregressionen im orthotopen Tumormodell festgestellt werden. Interessanterweise waren alle der überlebenden Tiere vor einer *re-challenge* mit PancOVA Tumoren und einige sogar mit Wildtyp Panc02 Tumoren (ohne OVA Expression) geschützt. Dieses Ergebnis ließ darauf schließen, dass es durch die Immuntherapie zu einem T-Zell Gedächtnis mit *epitope spreading* gekommen war. Durch den Nachweis p15E-spezifischer CTL im Blut der Langzeitüberlebenden (es handelt sich bei p15E um ein spezifisches Tumorantigen von Panc02 Zellen) konnte diese Hypothese bestätigt werden. Die Ergebnisse zeigen eine effiziente Methode, tumorinduzierte Immunsuppression durch geeignete immuntherapeutische Strategien zu überwinden. Das Konzept der Tumorvakzine mit unterschiedlichen Tumorantigenen wird in der Therapie des Pankreaskarzinoms alleine in den USA aktuell in über zehn klinischen Studien untersucht (http://www.cancer.gov/clinicaltrials/search/results?protocolsearchid=11444759, 28.02.2013). Die Verwendung von ISCOM-Vakzinen zusammen mit TLR Liganden könnte hier in Zukunft einen entscheidenden Vorteil bringen.

1.5.1.3 Ellermeier et al. Cancer Res 2013.

Therapeutic efficacy of bifunctional siRNA combining TGF-β_1 silencing with RIG-I activation in pancreatic cancer.

Der Hauptteil meiner Arbeit befasst sich mit der Therapie des Pankreaskarzinoms mittels einer bi-funktionalen, RIG-I aktivierenden siRNA gegen TGF-β. TGF-β ist einer der zentralen Treiber Pankreaskarzinom-induzierter Immunsuppression. RIG-I ist eine zytosolische Helikase, die virusassoziierte 5‘-Triphosphat-RNA erkennt und nach Aktivierung zu einer anti-viralen Typ 1 IFN Antwort führt sowie Apoptose induziert. Die Kombination aus RNA-Interferenz und RIG-I-Aktivierung konnte durch eine Triphosphatmodifikation am 5‘-Ende der TGF-β-spezifischen siRNA (ppp-TGF-β) mittels *in-vitro*-Transkription eines entsprechenden DNA-Templates erreicht werden. Eine auf ähnliche Weise generierte ppp-siRNA gegen Bcl-2, mit dem Ziel

Apoptoseinduktion zu verstärken, hatte sich in einer Arbeit von Poeck et al. (2008) prinzipiell als erfolgreich in einem murinen Melanommodell erwiesen.

Wir konnten zeigen, dass humane Pankreaskarzinomzellen funktionelles RIG-I exprimieren und somit für eine ppp-RNA-Therapie in Frage kommen. Die von uns entwickelte ppp-TGF-β führte sowohl in murinen als auch in humanen Tumorzellen zu einer signifikanten Genexpressionshemmung von TGF-β, zur Produktion pro-inflammatorischer Zytokine und Chemokine (IFN-β, CXCL10) sowie zu Apoptose *in vitro*. Die Bifunktionalität konnte auch *in vivo* bestätigt werden. In einem murinen, orthopen Pankreaskarzinommodell kam es nach intravenöser Gabe von ppp-TGF-β zur Reduktion der TGF-β-Spiegel im Serum und Tumorgewebe, zu systemischer Immunaktivierung (pro-inflammatorische Zytokine, Immunzellaktivierung) sowie verstärkter Einwanderung von $CD8^+$ T-Zellen in das Tumorgewebe. Ferner zeigte sich eine Caspase-9-vermittelte Apoptose von Tumorzellen. Durchflusszytometrische Analysen der Immunzellinfiltrate im Tumor und lymphatischen Organen zeigten eine Aktivierung von $CD8^+$ T-Zellen sowie eine Reduktion von MDSC, die zudem einen Phänotyp verminderter Suppressivität aufwiesen. Hinweise auf Organschäden oder durch die Therapie induzierte Autoimmunprozesse fanden sich nicht. Überlebensversuche zeigten einen signifikanten Therapieerfolg der mit ppp-TGF-β behandelten Versuchstiere, wobei es in 33% der Fälle zu einer kompletten Remission kam, wie Autopsien der überlebenden Tiere nach 100 Tagen ergaben. Versuche mit Immunzell-depletierenden Antikörpern konnten belegen, dass der Therapieerfolg auf der Aktivierung tumorreaktiver $CD8^+$ T-Zellen beruhte, während NK-Zellen entbehrlich waren. Zusammenfassend konnten wir zeigen, dass der Einsatz einer bi-funktional wirksamen, immunstimulatorischen siRNA gegen TGF-β ein innovatives, sicheres und vielversprechendes Therapiekonzept darstellt.

1.5.2 *Summary of the presented publications*

Over the course of the past decades major progress has been made regarding malignoma therapies and outcomes. Tumor immunotherapy has been established as the fourth pillar of tumor therapy. Nevertheless, patients with certain malignancies such as pancreatic carcinoma have hardly been able to benefit from new therapeutic regimens. Tumor-mediated immunosuppression is seen as a major reason for this lack of treatment efficacy. Therefore continuous effort is needed to shed more light on the mechanisms of tumor-mediated immunosuppression and to develop promising therapeutic strategies which are suited to eventually enter clinical application.

1.5.2.1 Anz et al. Int J Cancer 2011.

CD103 is a hallmark of tumor-infiltrating regulatory T-cells.

The first part of my thesis deals with the role of the $CD103^+$ (cluster of differentiation 103, also known as αEβ7) subpopulation of regulatory T-cells (Treg). Treg play a crucial role in tumor-mediated immunosuppression. Anti-Treg agents already in use or theoretically plausible exhibit major drawbacks. The target structures are either hardly accessible due to intracellular localization (FoxP3), therapeutic efficacy plummets while tumor load increases (CD25) or major side effects of autoimmunity occur due to unspecificity (CTLA4) (Onizuka et al. 1999, Kapadia et al. 2005, Colombo et al. 2007).

First, we were able to confirm the increased immunosuppressive function of $CD103^+$ as compared to $CD103^-$ Treg. By analyzing four different murine tumor models (EL4 lymphoma, CT26 colon carcinoma, B16 melanoma and Panc02 pancreatic carcinoma) we were able to show that $CD103^+$ is upregulated in tumor infiltrating Treg. Treg retention in the tumor stroma however was not mediated by CD103. Our results further indicated that TGF-β is the key player in the process of induction of the $CD103^+$ subpopulation since TGF-β knockdown via RNA interference in a model of murine orthotopic pancreatic carcinoma led to significantly reduced numbers of $CD103^+$ Treg in the tumor tissue. In conclusion we can state that CD103 and its regulation by TGF-β are potential therapeutic targets to break tumor-mediated immunosuppression.

1.5.3.2 Jacobs et al. Int J Cancer 2011.

An ISCOM vaccine combined with a TLR9 agonist breaks immune evasion mediated by regulatory T-cells in an orthotopic model of pancreatic carcinoma.

In the second part of my thesis we used an ovalbumine (OVA)-expressing model of murine, orthotopic Panc02 pancreatic carcinoma to test the therapeutic efficacy of an ISCOMATRIX (IMX)-based vaccine containing the model antigen OVA (OVA/IMX). IMX consists of cholesterol, saponin and phoshpolipids and forms particles of approximately 40 nm in diameter. IMX-based vaccines, which contain the adjuvant plus a protein antigen, have been demonstrated to efficiently induce both humoral and adaptive immune responses to vaccine antigen. IMX has been shown to activate DC *in vivo* and to facilitate antigen cross-presentation by DC (Davis et al. 2004, Schnurr et al. 2005, Drane et al. 2007, Schnurr et al. 2009, Duewell et al. 2011). To counteract immunosuppressive mechanisms derived from the tumor we evaluated the IMX-based vaccine alone, in combination with a TLR9 agonist (CpG-ODN 1826) or with a depleting antibody against CD25.

Prophylactic vaccination with OVA/IMX led to effective tumor protection via induction of antigen-specific CTL. In a therapeutic setting, when tumors were already established, OVA/IMX did not prove sufficiently effective, partly due to tumor-mediated induction of Treg. The addition of an anti-CD25 antibody significantly improved therapy outcomes. The combination of the vaccine with the TLR9 agonist led to a Th1-dominant immune response along with the activation of the adaptive immune system, especially $CD8^+$ T cells. Significantly prolonged survival rates including several long-term survivors could be achieved in the orthotopic PancOVA model of murine pancreatic cancer. Strikingly, those long-term survivors all rejected PancOVA tumors and partially rejected Panc02 wildtype cancer cells lacking OVA-expression in re-challenge experiments. We assumed this to be due to T-cell memory induction and epitope spreading. Our hypothesis was supported by isolating p15E-specific CTL in the blood of long-term survivors (the p15E epitope is derived from a tumor antigen expressed by Panc02 pancreatic cancer cells). The results show a potent strategy to break tumor-induced immunosuppression. In the USA alone, there are currently more than ten clinical trials ongoing, testing vaccination strategies for the treatment of pancreatic carcinoma (http://www.cancer.gov/clinicaltrials/search/results?protocolsearchid=11444759, February 28, 2013). The use of IMX vaccines in combination with TLR ligands or other immune modifiers could potentially create a significant treatment advantage in the future.

1.5.3.3 Ellermeier et al. Cancer Res 2013.

Therapeutic efficacy of bifunctional siRNA combining TGF-β_1 silencing with RIG-I activation in pancreatic cancer.

The main part of my work focused on the development of a bi-functional, RIG-I activating siRNA targeting TGF-β for immunotherapy of pancreatic carcinoma. TGF-β is a key promoter of cancer-induced immunosuppression, which is highly overexpressed by pancreatic cancer cells. RIG-I is a cytosolic helicase which detects virus-associated 5'triphosphate-RNA (ppp-RNA) and leads to an anti-viral type 1 IFN response as well as IFN- and p53-independent apoptosis. The combination of RNA interference with RIG-I activation in one RNA molecule was reached via 5'-triphosphate modification of a TGF-β-specific siRNA by *in vitro* transcription of a corresponding DNA-template (ppp-TGF-β). A related ppp-modified siRNA targeting the anti-apoptotic molecule Bcl-2, which was designed to enhance apoptosis induction in tumor cells, has demonstrated efficacy in a proof-of-principle study in a mouse model of malignant melanoma (Poeck et al. 2008).

We were able to show that human pancreatic cancer cells express functional RIG-I and hence are susceptible to ppp-RNA therapy. Bifunctional ppp-TGF-β led to significant TGF-β knockdown, production of pro-inflammatory cytokines and apoptosis in murine as well as human pancreatic cancer cells *in vitro*. Following intravenous ppp-TGF-β treatment in an orthotopic model of murine Panc02 pancreatic carcinoma, TGF-β suppression systemically as well as in the tumor tissue was achieved. Furthermore, treatment led to systemic immune activation (production of pro-inflammatory cytokines, activation of immune cells), increased migration of $CD8^+$ T-cells to the tumor tissue and caspase 9-mediated tumor cell apoptosis. MDSC in tumor-bearing mice were reduced in number and showed a less suppressive phenotype. Treatment-related toxicity or autoimmunity was not detected. Survival experiments showed a significant benefit with complete tumor remission in 33% of all ppp-TGF-β treated animals. Therapeutic efficacy was significantly better for the bi-functional siRNA molecule as compared to RNA molecules mediating either RIG-I activation or TGF-β gene silencing alone. Therapeutic efficacy strongly relied on $CD8^+$ T-cells, whereas NK cells appeared to be dispensable, as evidenced with immune cell depleting antibodies. In conclusion we were able to show that RIG-I is a promising target in pancreatic cancer and that bi-functional immunostimulatory ppp-siRNA targeting TGF-β is an innovative, safe and promising therapeutic concept.

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3. Abkürzungsverzeichnis

APC	Antigen-präsentierende Zelle
ATP	Adenosin-Triphosphat
BCL-2/xL	*B-cell lymphoma* 2/xL
CD	*Cluster of differentiation*
CpG-ODN	Cytosin-Phosphat-Guanin-Oligodesoxynukleotid
CTL	Zytotoxischer T-Lymphozyt
CXCL10	*C-X-C motif chemokine* 10
DAMP	*Danger-associated molecular pattern*
DC	Dendritische Zelle
DNA	Desoxyribonukleinsäure
EMT	*Epithelial-to-mesenchymal transition*
IFN-α/β/γ	Interferon alpha/beta/gamma
Ig	Immunglobulin
IL	Interleukin
IPS-1	*Interferon promoter stimulator* 1
IRAK	IL-1R-assoziierte Kinase
IRF	*IFN regulatory factor* 3
ISCOM	*Immuno stimulating complex*
LGP-2	*Laboratory of genetics and physiology* 2
MAMP	*Microorganism-associated molecular pattern*
MAP-Kinasen	*Mitogen-activated protein* Kinasen
MDA-5	*Melanoma differentiation gene* 5
MDSC	*Myeloid derived suppressor cell*
MHC	*Major histocompatibility complex*
MyD88	*Myeloid differentiation factor* 88
NF-κB	*Nuclear factor kappa B*
NK-Zelle	Natürliche Killerzelle
OVA	Ovalbumin
PAMP	*Pathogen-associated molecular pattern*
ppp-RNA	Triphosphat siRNA
PRR	*Pattern-recognition*-Rezeptor
RIG-I	*Retinoic acid inducible gene* I
RNA	Ribonukleinsäure
SMAD7	*Mothers against decapentaplegic homolog* 7
TGF-β	*Transforming growth factor beta*
TGF-βR	*Transforming growth factor beta* Rezeptor

TLR	*Toll-like* Rezeptor
TNF-α	*Tumor necrosis factor alpha*
TRIF	*Toll/IL-1R domain-containing adapter inducing IFN-*

4. Ergebnisse

4.1 Originalarbeit: Anz D, et al. Int J Cancer 2011

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CD103 is a hallmark of tumor-infiltrating regulatory T cells

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Regulatory T cells (Treg) mediate tolerance towards self-antigens by suppression of innate and adaptive immunity. In cancer patients, tumor-infiltrating FoxP3+ Treg suppress local anti-tumor immune responses and are often associated with poor prognosis. Markers that are selectively expressed on tumor-infiltrating Treg may serve as targets for immunotherapy of cancer. Here we show that CD103, an integrin mediating lymphocyte retention in epithelial tissues, is expressed at high levels on tumor-infiltrating FoxP3+ Treg in several types of murine cancer. In the CT26 model of colon cancer up to 90% of the intratumoral FoxP3+ cells expressed CD103 compared to less than 20% in lymphoid organs. CD103+ Treg suppressed T effector cell activation more strongly than CD103^{neg} Treg. Expression of CD103 on Treg closely correlated with intratumoral levels of transforming growth factor β (TGF-β) and could be induced in a TGF-β-dependent manner by tumor cell lines. *In vivo*, gene silencing of TGF-β reduced the frequency of CD103+ Treg, demonstrating that CD103 expression on tumor-infiltrating Treg is driven by intratumoral TGF-β. Functional blockade of CD103 using a monoclonal antibody did however not reduce the number of intratumoral Treg, indicating that CD103 is not involved in homing or retention of FoxP3+ cells in the tumor tissue. In conclusion, expression of CD103 is a hallmark of Treg that infiltrate TGF-β-secreting tumors. CD103 thus represents an interesting target for selective depletion of tumor-infiltrating Treg, a strategy that may help to improve anti-cancer therapy.

Regulatory T cells (Treg) are crucial in the prevention of autoimmunity by inhibiting effector T cell responses against self-antigens.[1] Treg however also inhibit immune responses against malignant tumors and thus facilitate cancer development.[2] Indeed, a prominent role of Treg in tumor-associated immunosuppression has been confirmed by several recent studies. During tumor progression Treg accumulate in the blood and lymphoid organs of the tumor-bearing host and in several types of cancer Treg abundantly infiltrate the tumor tissue itself.[2,3] Inhibition of anti-cancer immunity is mediated predominantly by tumor-infiltrating Treg that suppress effector T cell responses locally at the tumor site.[4] The number of tumor-infiltrating FoxP3+ Treg is associated with poor prognosis and has been identified as a significant predictor of patient death in several types of human cancer.[5–7]

Given the detrimental role of Treg in tumor progression, efforts were made to identify target molecules to selectively deplete these cells. The transcription factor FoxP3, the most distinctive marker characterized so far for Treg in both humans and mice, is not accessible to depleting antibodies due to its intracellular expression.[3] Natural thymus-derived Treg constitutively express the interleukin-2 receptor α-chain (CD25) and treatment of mice with monoclonal antibodies against CD25 leads to a temporary reduction of CD4+FoxP3+ cells.[8] This enhances anti-tumor immunity and can lead to T cell dependent rejection of pre-existing tumors.[9] However, with tumor progression the efficacy of anti-CD25 treatment is gradually reduced, a fact that may result from simultaneous depletion of activated CD25-expressing effector T cells.[10] Another antibody-mediated strategy to inhibit Treg function is the activation or blockade of target molecules on these cells without depletion. Activation of the glucocorticoid-induced tumor-necrosis factor receptor related protein (GITR) by an agonistic antibody inhibits Treg function and shows *in vivo* anti-tumor activity.[11,12] A blocking antibody to the cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) expressed by both regulatory and effector T cells inhibits Treg-induced suppression and is currently evaluated in clinical trials.[3,13] Anti-CTLA4 treatment however affects the entire pool of Treg and an important limitation of this approach is the development of systemic autoimmunity.[14] A marker predominantly expressed by

Key words: CD103, regulatory T cell, FoxP3, tumor
Abbreviations: CTLA4: cytotoxic T-lymphocyte-associated antigen 4; TGF-β: transforming growth factor β; Treg: regulatory T cell
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tumor-infiltrating Treg would represent a more selective target to enhance anti-cancer immunity.

The plasma membrane-associated molecule CD103, also called $\alpha_E\beta_7$, belongs to the family of integrins and is poorly expressed by immune cells in the spleen or the peripheral lymph nodes.[15] CD103 can be detected mainly on T cell populations within the mucosal epithelium of the gut and on intestinal lamina propria leukocytes.[16] It is thought that CD103 contributes to the retention of lymphocytes in epithelial tissues through interaction with its receptor E-cadherin expressed by epithelial cells.[17] Mice deficient for CD103 have slightly reduced numbers of intestinal intraepithelial lymphocytes, but apart from that are healthy, indicating that CD103 is probably dispensable.[18] Among Treg in lymphoid tissues, a subset of about 20% expresses CD103 and these cells display an effector memory phenotype with low expression levels of CD45RB and high levels of CD44.[19] Some reports further indicate that CD103+ Treg more strongly inhibit CD4 T cell proliferation than conventional Treg.[19,20] Thus, CD103 is a surface-expressed molecule that marks both intestinal lymphocytes and a particularly suppressive subtype of Treg.

In this study, we analyzed tumor-infiltrating Treg for expression of CD103 in four different murine models of cancer. In all tumor models, we found that the majority of intratumoral Treg express CD103 with up to 90% of FoxP3+ Treg staining positive for CD103. High proportions of CD103+ cells were further specific for tumor-infiltrating Treg. CD103 expression on Treg correlated with TGF-β secretion of tumor cells and could be down-regulated by RNAi-mediated gene silencing of TGF-β. Therapeutic targeting of CD103 may represent a promising approach to enhance anti-cancer immunity.

Material and Methods

Mice and cell lines

Female BALB/c and C57BL/6 mice were purchased from Harlan-Winkelmann (Borchen, Germany). Mice were 5 to 10 weeks of age at the onset of experiments. Animal studies were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany). The murine cell lines Colon-26 (CT26; Cell Lines Service, Heidelberg, Germany), B16 melanoma F1 (LGC Promochem, Teddington, UK), EL-4 lymphoma (Institute of Immunology, University of Munich) and Panc02 (kindly provided by Prof. C. Bruns, Department of Surgery, University of Munich) were maintained in DMEM medium supplemented with 10% FCS, 1% L-glutamine, 1 U/ml penicillin and 0.1 mg/ml streptomycin (all PAA Laboratories, Coelbe, Germany). For tumor induction 0.25×10^6 (CT26), 1×10^6 (B16 and Panc02), or 5×10^6 (EL4) tumor cells were injected subcutaneously into the flank. The tumor-draining lymph nodes were identified by connecting vessels, the proximity to the tumor and the larger size compared to non-draining lymph nodes. Tumor size was expressed as the product of the perpendicular diameters of individual tumors (mm^2). Tumor growth was calculated as final tumor size divided by the number of days since injection (mm^2/d) to normalize data from independent experiments.

Immunohistology

Tumor tissues were frozen in liquid nitrogen and 5 μm cryosections from the center of the tumors were prepared. The following primary antibodies were used: anti-mouse CD103 (Biolegend, San Diego, CA), anti-mouse FoxP3 (Ebioscience, San Diego, CA) and anti-mouse E-Cadherin (Cell Signaling Technology, Beverly, MA). Cy5 F(ab)2 goat anti-Armenian hamster IgG, biotin F(ab)2 donkey anti-rat IgG, biotin IgG donkey anti-rabbit IgG and rhodamin red X streptavidin were used as detection reagents. Nucleic counterstaining was performed using DAPI (Sigma Aldrich, Steinheim, Germany). Counting was performed blinded by two independent investigators. Images were obtained by fluorescence microscopy (Axiovert 2000 Carl Zeiss, Jena, Germany; 40-fold magnification) using Carl Zeiss Axiovision software and processed with Adobe Photoshop for adjustment of contrast and size.

Flow cytometry

For flow cytometry analysis single cell suspensions of spleen, lymph nodes or Peyer's patches were prepared. Bone marrow cells were harvested from murine femur and tibia and erythrocytes were lysed with ammonium chloride buffer (BD Biosciences). To isolate lymphocytes from tumor, lung, liver or heart, the tissues were mechanically disrupted, incubated with 1 mg/ml collagenase and 0.05 mg/ml DNAse (both Sigma Aldrich) and subsequently passed through a cell strainer. Single cell suspensions were resuspended in 44% Percoll (Biochrome, Berlin, Germany) and layered over 67% Percoll prior to centrifugation at 800 g for 30 min. Lymphocytes from the interphase were stained for flow cytometry. The following antibodies were used: Pacific Blue or PerCP anti-mouse CD3, PE anti-mouse B220, PE-Cy7 or PerCP anti-mouse CD4, APC-Cy7 anti-mouse CD8 (all Biolegend), FITC anti-mouse CD103 (BD Biosciences, Heidelberg, Germany) and Pacific Blue or APC anti-mouse FoxP3 (Ebioscience). Intracellular detection of FoxP3 was performed using premixed regulatory T cell staining reagents (Ebioscience). Events were measured on a FACS Calibur or FACS Canto II flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, OR).

FACS sorting and proliferation assays

Untouched CD4+ T cells were sorted from single cell suspensions of lymph nodes by magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were stained with labeled antibodies against CD103 (Fitc, BD Biosciences), CD4 (PerCP) and CD25 (APC, both from Biolegend). CD4+ CD25+ CD103+, CD4+CD25+CD103^{neg} and CD4+ CD25^{neg} cells were obtained by FACS sorting using a FACS Aria cell sorter (BD Biosciences) with a purity of more than 99%. Treg subsets (3×10^4 cells) and T effector cells (7.5×10^4 cells) were cultured in triplicate with anti-CD3-CD28 beads (Invitrogen, Carlsbad, CA) at a bead-to-cell ratio of 1:5

for 72 hr and in the presence of BrdU (Roche Diagnostics, Mannheim, Germany). To detect proliferation of T effector cells, co-cultures were then stained with Pacific Blue-labeled FoxP3 antibody, fixed with 1% PFA in PBS, incubated with DNAse I (0.05 mg/ml in PBS; Sigma-Aldrich, Steinheim, Germany) and further stained with anti-BrdU-FITC antibody (Invitrogen). T effector cell proliferation and activation was determined by BrdU and CD69 expression of CD4+FoxP3^{neg} cells. IL-2 levels in the co-culture were measured by ELISA (BD Biosciences).

TGF-β in vitro assays

To assess CD103 induction on Treg, 1.2×10^5 splenocytes were cultured in triplicate with supernatants of CT26 or EL-4 tumor cells in the presence of anti-CD3-CD28 coated microbeads (Invitrogen, bead to cell ratio 1:10). Recombinant TGF-β1 or anti-TGF-β1 antibody (both R&D Systems, Minneapolis, MN) were added in a concentration of 5 ng/ml and 12.5 μg/ml, respectively. Cells were cultured for three days before analysis by flow cytometry.

TGF-β ELISA of supernatants and tissue lysates

To measure TGF-β secretion by different tumor cell lines, 5×10^6 tumor cells were plated in 3 ml of medium, cultured for two days and supernatants were analyzed by ELISA (R&D Systems). For analysis of tissues, tumor or lymph node homogenates were resuspended in lysis buffer (BioRad Laboratories, Hercules, CA) and centrifuged. Total protein concentration was measured by Bradford assay (BioRad Laboratories). All samples were diluted to equal protein concentrations and TGF-β1 levels were measured by ELISA. The final cytokine concentration was calculated as ng cytokine/g protein in the respective lysate.

TGF-β gene silencing and in vivo CD103 blocking

For *in vivo* gene silencing of TGF-β, siRNAs were designed according to published guidelines.[21] 3′-Overhangs were carried out as two deoxythymidine residues (dTdT). RNAs were all from Eurofins MWG Operon (Penzberg, Germany). Sequences were: Control RNA: 5′-GAUGAACUUCAGGGUCAGCG-3′ (sense), 5′-CGCUGACCCUGAAGUUCAUC-3′ (antisense); TGF-β1 siRNA: 5′-GAACUCUACCAGAAAUAUAUU-3′ (sense), 5′-AAUAUAUUUCUGGUAGAGUUC-3′ (antisense). Nonsilencing siRNA (control RNA) was designed to contain random sequences that do not match within the murine or human genome. For *in vivo* delivery 50 μg of siRNA was complexed with *in Vivo* JetPEI reagent (Peqlab, Erlangen, Germany) according to the manufacturer's instructions and injected into the tail vein. For *in vivo* blocking of CD103, 150 μg of rat IgG2aκ anti-mouse CD103 antibody (clone M290, Bioxcell, West Lebanon, NH) was injected intraperitoneally.

Statistics

All data are presented as mean +/− SEM and were analyzed as appropriate by unpaired Student's *t*-test or by ANOVA test using the Student Newman Keuls test. Statistical analysis was performed using SPSS software.

Results

A high proportion of tumor-infiltrating FoxP3+ regulatory T cells expresses CD103

To investigate CD103 expression levels on tumor-infiltrating Treg, subcutaneously induced murine CT26 colon and B16 melanoma tumors were examined by immunofluorescence staining. Tissue sections were double-stained with antibodies directed against FoxP3 and CD103 and the proportion of CD103+ Treg was evaluated by counting non-overlapping visual fields from tumors of eleven different mice (Fig. 1*a*). Strikingly, in CT26 tumors more than 90% of the tumor-infiltrating FoxP3+ cells expressed the integrin CD103 (Fig. 1*b*). In contrast, in the spleen only 20% of FoxP3+ cells were positive for CD103, consistent with previous reports.[22] In subcutaneous B16 melanoma tumors, more than 50% of intratumoral Treg expressed CD103 (Fig. 1*b*). We further used flow cytometry to analyze CD103 levels on tumor-infiltrating FoxP3+ cells and could confirm the high proportion of CD103+ Treg in both CT26 and B16 tumors (Fig. 1*c*). Analysis of an additional tumor model, the subcutaneous EL-4 lymphoma, revealed a lower percentage of CD103+ cells within tumor-infiltrating Treg (31%) compared to the CT26 and B16 tumors. In all analyzed models, however, CD103 expression of intratumoral Treg was significantly higher compared to FoxP3+ cells in the spleen of the same mice (Fig. 1*c*). Further, expression levels of CD103 by tumor-infiltrating Treg clearly correlated with tumor growth (Fig. 1*d*). To confirm previous reports indicating that CD103+ cells represent a particularly suppressive subset of Treg[19,20] we isolated CD103+ and CD103^{neg} Treg from CT26 tumor-bearing mice. Indeed, CD103+ Treg more strongly suppressed proliferation, activation and IL-2 release of T effector cells (Fig. 1*e*). In conclusion, tumor-infiltrating Treg are characterized by high expression levels of CD103, a marker predicting potent suppressive function of these cells.

High expression of CD103 is specific for tumor-infiltrating regulatory T cells

To assess whether the high proportions of CD103+ cells are specific for tumor-infiltrating Treg, we determined the percentage of CD103+ cells within FoxP3+ cells derived from different organs of tumor-bearing mice. In the peripheral lymph nodes, numbers of CD103+ cells among Treg were generally low with a proportion of less than 25% (Fig. 2*a*). Interestingly, in CT26 tumor-bearing mice a significantly higher number of CD103-expressing Treg was detected in the tumor-draining lymph nodes. To assess CD103 expression in

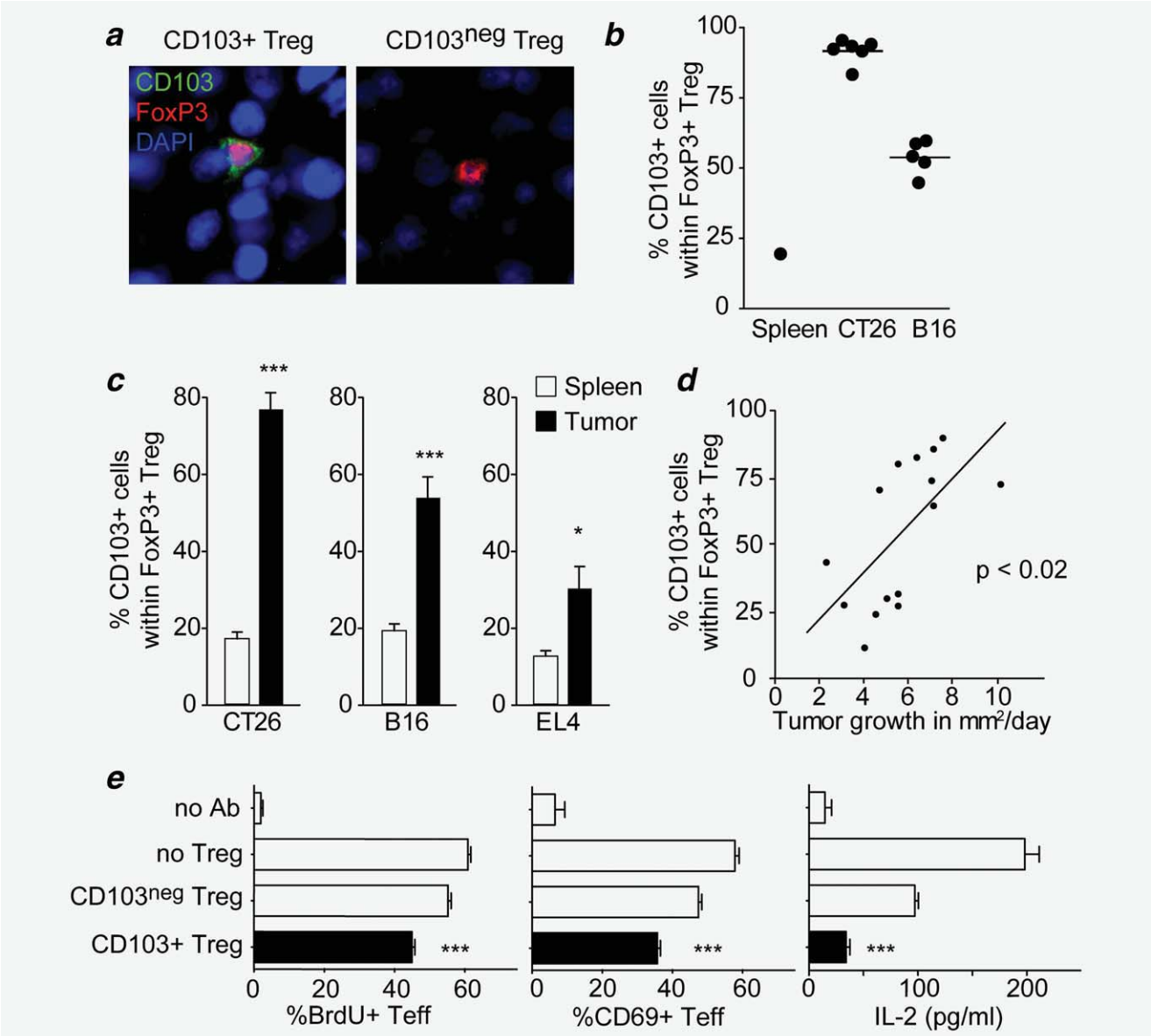

Figure 1. CD103 is highly expressed by tumor-infiltrating Treg. CT26, B16, and EL-4 tumors were induced by subcutaneous injection of the respective tumor cell line. Tumors were isolated and analyzed by immunohistology or flow cytometry when they reached a mean size of 100 mm^2. (*a*) Tissue sections of CT26 tumors were stained for CD103 (green) and FoxP3 (red) and representative images of CD103+ and $CD103^{neg}$ Treg are shown. (*b*) The proportion of CD103+ cells within FoxP3+ cells was determined in CT26 ($n = 6$) and B16 tumors ($n = 5$) and in the spleen of a C57BL/6 mouse ($n = 1$). Each data point represents the proportion of CD103+ cells within FoxP3+ cells in the tumor or the spleen of one mouse. (*c*) Expression of CD103 was determined by flow cytometry on CD4+FoxP3+ cells isolated from the spleen and the tumor tissue of CT26 ($n = 6$), B16 ($n = 5$), or EL-4 ($n = 6$) tumor-bearing mice. The mean percentage of CD103+ cells among CD4+FoxP3+ cells for all mice is shown. (*d*) Tumor growth of CT26 tumors in mm^2/day from three independent experiments was correlated to CD103 expression of CD4+FoxP3+ tumor-infiltrating cells according to Pearson's test. (*e*) CD4+CD25+CD103+ and $CD4+CD25+CD103^{neg}$ Treg as well as $CD4+CD25^{neg}$ T effector cells were isolated by FACS sorting from the tumor-draining lymph nodes of CT26 tumor-bearing mice. T effector cells were co-cultured with either Treg subset in the presence of anti-CD3-CD28 antibody. Proliferation and activation of T effector cells was measured by incorporation of BrdU, expression of CD69 and secretion of IL-2 using flow cytometry or ELISA. Error bars indicate SEM. *P* values for (*b* and *c*) were calculated relative to the proportion of CD103+ cells in the spleen and for (*e*) relative to suppression by $CD103^{neg}$ Treg (*$p < 0.05$; ***$p < 0.001$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

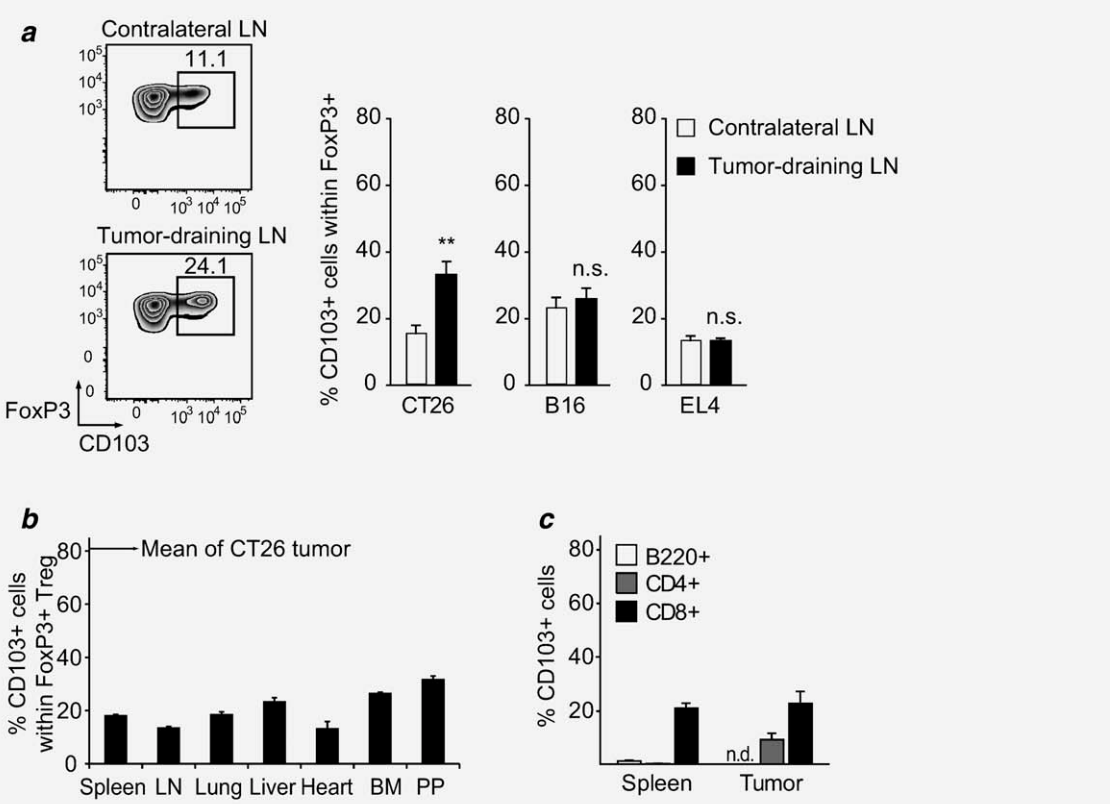

Figure 2. High expression of CD103 is characteristic for tumor-infiltrating Treg. (*a*) Subcutaneous tumors were induced as described in Figure 1 and lymph nodes were isolated when tumors reached a mean size of 100 mm^2. Expression of CD103 on CD4+FoxP3+ cells derived from both tumor-draining and contralateral lymph nodes was analyzed by flow cytometry (CT26, $n = 6$; B16, $n = 5$ and EL-4, $n = 6$). *p* values were calculated relative to the proportion of CD103+ cells in the contralateral lymph node (**$p < 0.01$). (*b*) The bar diagram shows the proportion of CD103+ cells among CD4+FoxP3+ cells isolated from the indicated organs of healthy BALB/c mice ($n = 6$; LN, lymph nodes; BM, bone marrow and PP, Peyer's patches). (*c*) The proportion of CD103+ cells among B220+, CD4+FoxP3^{neg} and CD8+ cells derived form the spleen and the tumor of CT26-bearing mice was analyzed by flow cytometry. Error bars indicate SEM.

other compartments, we isolated lymphocytes from lung, heart, liver, Peyer's patches, and bone marrow and determined CD103 expression by Treg. In all organs analyzed, the proportion of CD103+ cells among Treg was comparable to those in the peripheral lymph nodes (Fig. 2*b*). To further determine the proportion of CD103+ cells among other lymphocyte subsets, we analyzed B220+, CD4+FoxP3^{neg}, and CD8+ cells. Expression of CD103 was observed only on a small proportion of all lymphocyte subtypes, both in the spleen and the tumor tissue (Fig. 2*c*). Thus, high numbers of CD103+ cells are specific for tumor-infiltrating Treg.

Expression of CD103 in tumor-infiltrating regulatory T cells is driven by intratumoral transforming growth factor β

We next examined the mechanism responsible for enhanced CD103 expression on tumor-infiltrating Treg. In gut-associated lymphoid tissue, CD103 expression by lymphocytes is known to be induced by transforming growth factor β (TGF-β)[16] and thus, we hypothesized that cancer-associated TGF-β may give rise to high CD103 levels on tumor-infiltrating Treg. As significant differences were seen in the proportion of CD103+ cells among Treg within different tumor models (Fig. 1*c*), we quantified tumor-associated TGF-β levels to establish a possible relation. Cell culture supernatants as well as tissue lysates of CT26, B16, and EL-4 tumors were analyzed by ELISA. Whereas high and intermediate levels of TGF-β were detected in the supernatants of CT26 and B16 cells, respectively, EL-4 tumor cells did not produce this growth factor (Fig. 3*a*). *In vivo*, a similar pattern of TGF-β levels was observed, with the lowest levels of this cytokine in EL-4 tumors (Fig. 3*b*). Both *in vitro* and *in vivo* levels of TGF-β correlated with CD103 expression by tumor-infiltrating Treg, with high levels of both parameters in CT26 and B16 tumors. As CD103+ Treg were more frequent in tumor-draining than in contralateral lymph nodes we also compared TGF-β levels in these organs. Indeed, in the tumor-draining lymph nodes we detected significantly higher amounts of TGF-β than in non-draining lymph nodes in all but one mouse, thus confirming the positive correlation with CD103 expression (Fig. 3*c*).

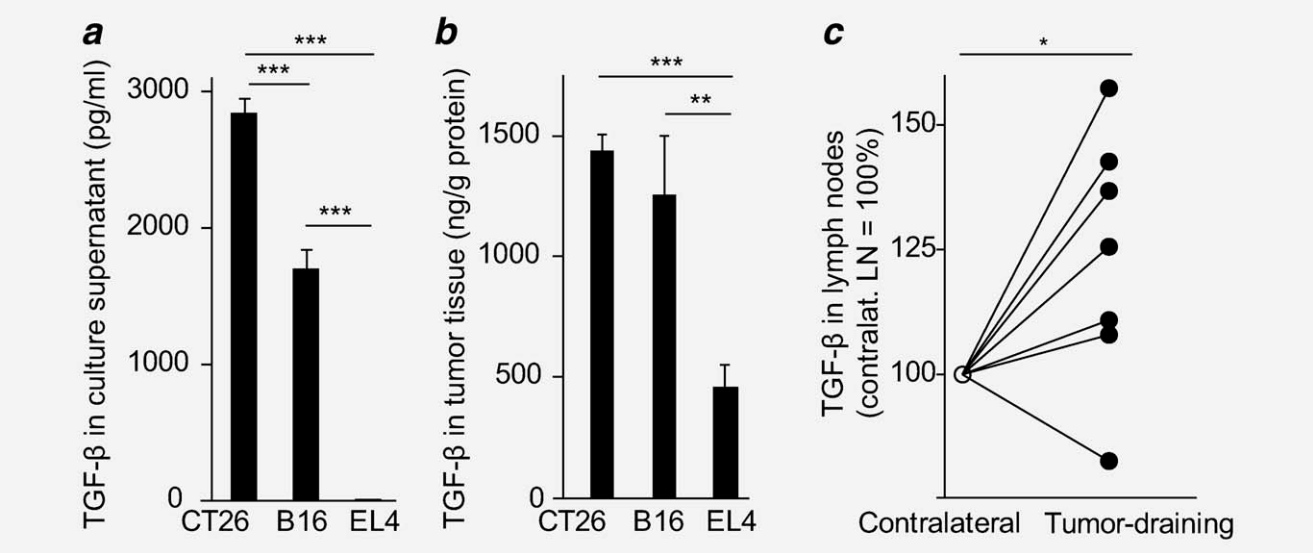

Figure 3. Tumor-derived TGF-β secretion correlates with CD103 expression by Treg. (*a*) Equal numbers of CT26, B16 and EL-4 tumor cells were cultured in triplicate for two days and TGF-β levels in the supernatant were determined by ELISA. (*b* and *c*) Subcutaneous tumors were induced as described in Figure 1 and isolated when they reached a mean size of 100 mm^2. TGF-β levels were determined by ELISA in tissue lysates of the respective tumors (CT26, $n = 6$; B16, $n = 4$ and EL-4, $n = 5$) and of the tumor-draining and contralateral lymph nodes derived from CT26-bearing mice. The concentration of TGF-β in the tumor-draining lymph nodes is shown relative to the contralateral lymph nodes (set at 100%) for each mouse. Error bars indicate SEM. *p* values were calculated as indicated by the lines (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

To investigate whether tumor-associated TGF-β triggers CD103expression by Treg, we cultured splenocytes in supernatants from CT26 tumor cells and quantified the proportion of CD103+ cells within CD4+FoxP3+ Treg. Indeed, CT26 supernatants induced CD103 expression by Treg and blocking of TGF-β efficiently prevented CD103 induction (Fig. 4*a*). In contrast to CT26, supernatants of TGF-β-negative EL-4 tumor cells did not induce CD103 on Treg. A clear up-regulation of CD103 on Treg was however observed upon addition of recombinant TGF-β to EL-4 supernatants (Fig. 4*b*) or by recombinant TGF-β alone (not shown). These data demonstrate that CD103 expression on Treg is directly triggered by tumor cell-derived TGF-β. Interestingly, this effect was specific for FoxP3+ Treg, as no induction of CD103 was seen on CD4+$FoxP3^{neg}$ or CD8+ T cells (Fig. 4*c*). The role of TGF-β in CD103 induction on Treg was further assessed *in vivo* by siRNA-mediated knock-down. Treatment of tumor-bearing mice with a TGF-β siRNA significantly reduced intratumoral levels of TGF-β, as determined by ELISA of tissue lysates (Fig. 4*d*). We then analyzed CD103 expression by tumor-infiltrating FoxP3+ cells using immunohistology. In mice treated with a control RNA nearly 50% of intratumoral Treg expressed CD103. Strikingly, a significant decrease of CD103 expression by Treg was observed upon treatment with the TGF-β-specific siRNA (Figs. 4*e* and 4*f*). In conclusion, expression of CD103 by tumor-associated Treg is driven by TGF-β.

CD103 is not required for the retention of regulatory T cells in the tumor

CD103 is an integrin that mediates retention of lymphocytes in epithelial tissues.[17] To assess whether expression of CD103 is necessary for the homing and retention of Treg in malignant tumors, we treated tumor-bearing mice with a blocking antibody against CD103. The monoclonal rat anti-mouse CD103 antibody (clone M290) binds to the α_E-subunit and blocks the interaction of CD103 with its receptor E-cadherin, but does not deplete CD103+ cells.[23,24] Treatment with anti-CD103 antibody was started one week after tumor induction and infiltration by FoxP3+ cells was evaluated 10 days later. We observed no differences in the number of tumor-infiltrating FoxP3+ cells between untreated and anti-CD103 treated mice (Fig. 5*a*). Efficient delivery of the antibody was confirmed by showing *in vivo* binding of M290 to CD103; this was demonstrated by staining frozen tumor sections of treated mice with a fluorescence-labeled anti-rat antibody (Fig. 5*b*). Anti-CD103 treatment further did not alter Treg or CD8 T cell numbers in the tumor-draining lymph nodes (Figs. 5*c* and 5*d*) or the spleen (data not shown) and had no impact on tumor growth (Fig. 5*e*). In addition, we found that the only known receptor for CD103, E-cadherin, is not expressed within CT26 tumors (Fig. 5*f*). Thus, although the majority of FoxP3+ cells expresses CD103, this integrin appears not to be required for the retention of Treg in the tumor tissue. Therapeutic targeting of CD103+ cells will therefore require the use of an antibody with depleting rather

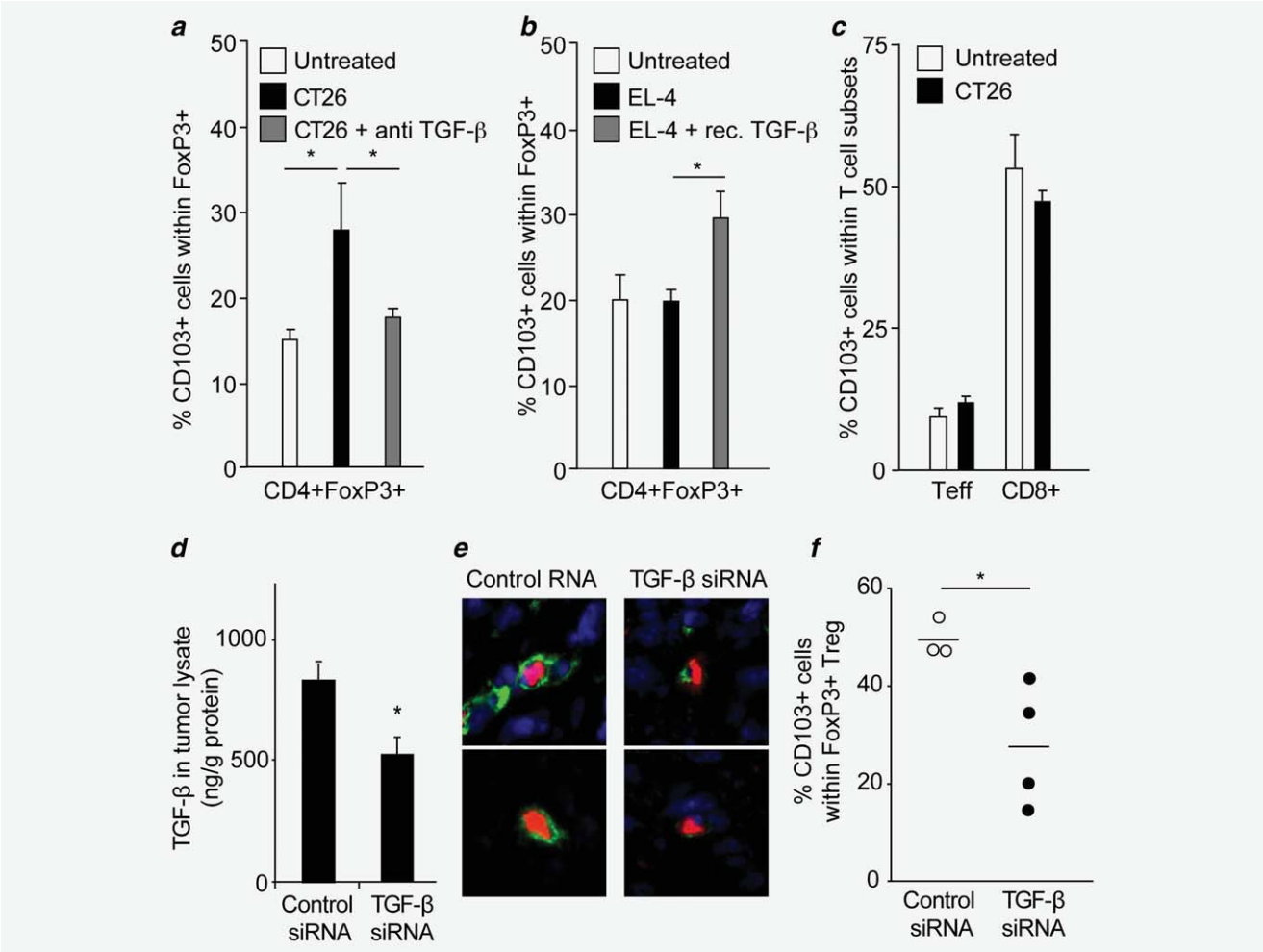

Figure 4. Expression of CD103 on tumor-infiltrating Treg is mediated by TGF-β. (*a–c*) Freshly isolated splenocytes (1.2×10^5) were stimulated with anti-CD3-CD28 coated microbeads and cultured with supernatants of CT26 or EL-4 tumor cells in the presence of either TGF-β blocking antibody or recombinant TGF-β. CD103 expression on CD4+FoxP3+, CD4+FoxP3neg (Teff) and CD8+ T cells was determined by flow cytometry after three days. (*d–f*) Subcutaneous Panc02 tumors of C57BL/6 mice were treated with a siRNA (50 μg i.v.) directed against TGF-β1 ($n = 4$) or with an irrelevant control RNA ($n = 3$) 10 days after induction. siRNA treatment was repeated after 36 hr and 12 hr later tumors were removed for analysis by ELISA and immunohistology. TGF-β levels were determined by ELISA in tissue lysates of RNA-treated tumors (*d*). Tumor sections were double-stained for CD103 (green) and FoxP3 (red) and two representative images are shown for both control RNA and TGF-β siRNA-treated mice (*e*). The proportion of CD103+ cells within FoxP3+ cells was determined in both groups by counting non-overlapping visual fields. Each data point represents the mean proportion of CD103+ cells within FoxP3+ Treg in the tumor of one mouse and bars indicate the mean of one treatment group (*f*). Error bars indicate SEM. *p* values were calculated relative to control RNA-treated mice (*$p < 0.05$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

than blocking function. The development of such an antibody could be a promising approach for cancer immunotherapy.

Discussion

Treg inhibit immune responses against malignant tumors and represent an important obstacle for cancer immunotherapy.[3] In particular, Treg infiltrating the tumor tissue itself inhibit anti-cancer immunity and correlate with poor prognosis in many types of human cancer.[4,5,25] A comprehensive knowledge of the phenotype of tumor-infiltrating Treg is crucial to understand their mode of action and to develop therapeutic strategies that target these cells. In this study, we demonstrate that the majority of tumor-infiltrating Treg expresses CD103, a cell surface protein of the integrin family. In CT26 tumors more than 90% of Treg expressed CD103 and a high proportion of CD103+ cells within intratumoral FoxP3+ Treg was observed in three other models of murine cancer. In contrast, analysis of CD103 expression by Treg in a broad panel of peripheral organs including the spleen, lymph nodes, lung, liver, heart, bone marrow, and Peyer's patches revealed

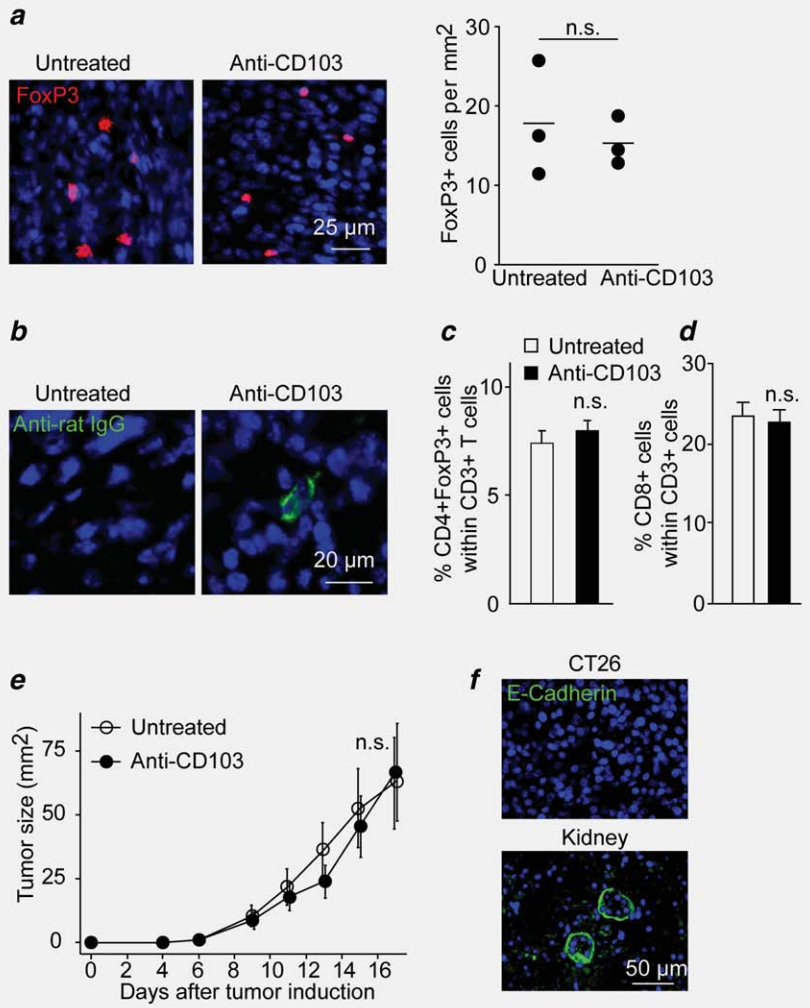

Figure 5. CD103 is not required for the retention of Treg in the tumor tissue. (*a–e*) Seven days after induction of subcutaneous CT26 tumors mice received intraperitoneal injections of 150 µg monoclonal rat anti-mouse CD103 antibody every second day (clone M290) or remained untreated ($n = 6$ for each group). Tumors were removed ten days after the first treatment and analyzed by immunohistology. (*a*) Representative images and quantification of tumor-infiltrating FoxP3+ cells ($n = 3$ for each group). (*b*) Frozen tissue sections of untreated and anti-CD103-treated tumors were stained with biotin-conjugated anti-rat IgG antibody, followed by fluorescence-conjugated streptavidin (green) for the *in vivo* detection of the monoclonal rat-anti mouse CD103 antibody. (*c* and *d*) The proportion of CD4+FoxP3+ cells and CD8+ cells within CD3+ cells in the tumor-draining lymph node was determined by flow cytometry. (*e*) Tumor growth of both untreated and anti-CD103 treated mice was determined. Error bars indicate SEM. *p* values were calculated relative to untreated mice (n.s., not significant). (*f*) Untreated tumor tissues of CT26 tumors (experiment described in Fig. 1) were analyzed by immunohistology for the expression of E-cadherin (green) and kidney sections from healthy mice were used as positive control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

only low expression levels with about 20% CD103+ cells among FoxP3+ Treg. Thus, in cancer-bearing hosts high expression of CD103 is a unique property of tumor-infiltrating Treg. Previous work has shown that positivity for CD103 defines a subset of Treg with specific migratory and anti-inflammatory properties: CD103+ Treg express a set of chemokine receptors similar to activated T cells and thus preferentially home to sites of inflammation.[22] The specific pattern of chemokine receptor expression could thus explain the accumulation of these cells in malignant tumors. An important property of CD103+ Treg is their highly suppressive function: CD25+CD103+ Treg inhibit effector T cell proliferation more potently than CD25+CD103^{neg} cells[19,20] and tumor-derived CD103+ Treg suppress CD8 T cell responses more strongly than CD103^{neg} Treg.[26] The prevalence of CD103+ Treg that we observed in several types of tumors may therefore enhance immunosuppression. We conclude that CD103 represents an interesting molecule to

therapeutically target a tumor-resident and highly immunosuppressive Treg subset.

Due to the central role of Treg in tumor-associated immunosuppression, targeting these cells by specific antibodies is a promising approach. Depleting antibodies against CD25 have been extensively tested and show some efficacy in mice, but lack significant benefits in patients so far.[27] A blocking antibody against CTLA-4, a receptor expressed by T cells, synergistically enhances anti-tumor immunity by inhibiting Treg-induced suppression and promoting T effector cell activation[28]; this treatment can however lead to systemic autoimmunity as CTLA-4 is ubiquitously expressed.[14] For CD103, we have shown predominant expression by Treg in the tumor tissue and to some extent in the tumor-draining lymph node. Thus, targeting this molecule could eliminate Treg at the sites where they most potently suppress tumor-specific immunity. Unfortunately, a depleting antibody against CD103 has so far not been developed. In mice, only antibodies of the rat IgG2b and to some extent of the IgG1 isotype bear the potential to induce antibody-mediated cytotoxicity leading to depletion of target cells.[29,30] In this study we used the monoclonal rat IgG2aκ antibody M290, which blocks the interaction of CD103 with its receptor E-cadherin.[23,24] As expected, M290 treatment did not deplete CD103+ cells. However, we speculated that anti-CD103 treatment could reduce Treg numbers *via* interfering with the retention of Treg within the tumor. The principal function of CD103 is to mediate adhesion of cells in epithelial tissues[17] and for Treg, it has been shown that CD103 is essential for the retention in inflamed skin during infection with the parasite Leishmania major.[31] As Treg numbers were not altered by anti-CD103 treatment, our data show that CD103 is not involved in the retention of Treg within malignant tumors. This is further supported by the lack of E-cadherin expression in the tumor tissue, the so far only identified ligand for CD103. Thus, blocking CD103 is not sufficient to suppress tumor infiltration by Treg, but generation of depleting antibodies will be an interesting approach for immunotherapy of cancer.

As CD103 does not mediate retention of Treg in the tumor tissue, another mechanism must be responsible for the accumulation of intratumoral FoxP3+ cells expressing this integrin. Our data suggest that intratumoral TGF-β promotes the expression of CD103 on tumor-infiltrating Treg. We found that high levels of CD103 were expressed predominantly in those types of tumors with strong TGF-β secretion. In addition, *in vivo* gene silencing of TGF-β reduced the number of intratumoral CD103+ Treg. Our hypothesis is supported by previous reports showing that TGF-β is a potent inducer of CD103 *in vitro*.[32,33] Further, *in vivo* TGF-β mediates expression of CD103 by intraepithelial and lamina propria-associated lymphocytes[16] and induces the generation of CD103-expressing FoxP3+ Treg from naïve T cells.[34] The high number of tumor-infiltrating CD103+ Treg could thus result from the conversion of previously nonregulatory T cells. In conclusion, CD103 represents a good marker to selectively target Treg in TGF-β-secreting tumors and the development of novel depleting antibodies against CD103 may be a promising approach to improve anti-cancer therapy.

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An ISCOM vaccine combined with a TLR9 agonist breaks immune evasion mediated by regulatory T cells in an orthotopic model of pancreatic carcinoma.

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An ISCOM vaccine combined with a TLR9 agonist breaks immune evasion mediated by regulatory T cells in an orthotopic model of pancreatic carcinoma

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Vaccines based on immune stimulatory complexes (ISCOM) induce T-cell responses against tumor antigen (Ag). However, immune responses are impaired in pancreatic cancer patients. We investigated the efficacy of an ISCOM vaccine in a murine pancreatic carcinoma model. Panc02 cells expressing OVA as a model Ag were induced subcutaneously or orthotopically in the pancreas of C57BL/6 mice. Treatment consisted of an OVA containing ISCOM vaccine, either used alone or in combination with the TLR9 agonist CpG. The ISCOM vaccine effectively induced Ag-specific CTL capable of killing tumor cells. However, in mice with established tumors CTL induction by the vaccine was inefficient and did not affect tumor growth. Lack of efficacy correlated with increased numbers of Treg. Depletion of Treg with anti-CD25 mAb restored CTL induction and prolonged survival. Adding low-dose CpG to the ISCOM vaccine reduced Treg numbers, enhanced CTL responses and induced regression of pancreatic tumors in a $CD8^+$ T cell–dependent manner. Mice cured from the primary tumor mounted a memory T-cell response against wild-type Panc02 tumors, indicative of epitope spreading. Combining ISCOM vaccines with TLR agonists is a promising strategy for breaking tumor immune evasion and deserves further evaluation for the treatment of pancreatic carcinoma.

Key words: tumor immunity, vaccination, dendritic cells, antigen presentation

Abbreviations: Ag: antigen; CpG: unmethylated cytosine/guanine oligodeoxynucleotide with a phosphodiester backbone; CTL: cytotoxic T lymphocytes; DC: dendritic cells; ISCOM: immune stimulatory complex; mAB: monoclonal antibody; OVA: ovalbumin; TLR: toll-like receptor; Treg: regulatory T cells

Additional Supporting Information may be found in the online version of this article.

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Pancreatic cancer is one of the most fatal malignancies in the Western world. It is the fourth leading cause of cancer death in the United States.[1] The 5-year survival rate is less than 5% with a median overall survival time of 3–6 months. Despite advancements made over the past two decades in elucidating molecular pathways involved in pancreatic carcinogenesis and in the field of targeted therapy, the clinical outcome has not yet significantly improved. At the time of diagnosis the majority of patients present with locally advanced, unresectable tumors or metastatic disease. Even in the small number of patients who undergo surgery in a curative intention most patients succumb to recurrent and metastatic disease. Therefore, new treatments are urgently needed.

Immunotherapy may offer a new treatment option. Pancreatic carcinoma cells can be recognized by T cells, which are found in the blood of pancreatic carcinoma patients.[2] Tumor infiltration with T cells represents a positive prognostic factor.[3] However, pancreatic carcinomas promote systemic and locally active immunosuppressive mechanisms.[4] These include inhibition of T-cell activation, secretion of immunosuppressive cytokines, defects in Ag presentation and recruitment of regulatory T cells (Treg), a subgroup of $CD4^+$ T cells with suppressor function.[5,6] In patients with pancreatic carcinoma, increased numbers of Treg are found in the

peripheral blood and within the tumor.[7] It has been shown that increased Treg prevalence is a negative prognostic factor in various malignancies, including pancreatic adenocarcinoma.[8–10] Therefore, breaking tumor-induced immunosuppression is a major challenge for immunotherapy.

Tumor vaccines aim at inducing CTL capable of recognizing and killing tumors. A prerequisite is crosspresentation of tumor Ag on MHC class I by DC. We previously reported that human DC pulsed with tumor cell lysate or apoptotic tumor cells induce CTL responses against pancreatic cancer cells *in vitro*.[11,12] In another study, we demonstrated that a DC vaccine combined with gemcitabine-based chemotherapy can achieve prolonged survival in an orthotopic model of pancreatic carcinoma.[13] Other groups have shown that intratumoral immunization with DC pulsed with tumor RNA or alpha-galactosylceramide can induce antitumor immunity in murine models of pancreatic cancer.[14,15] However, the production of DC vaccines is a time consuming and expensive process. A promising strategy is targeting vaccines to DC *in vivo*. The development of cell-free vaccines will make vaccines accessible to a larger number of patients and facilitate their evaluation in clinical trials. ISCOM vaccines combine an efficient Ag delivery system with the immune-stimulatory activity of saponin and have been shown to target DC *in vivo* and to promote humoral and cellular immune responses.[16] Ag can either be formulated into an ISCOM vaccine during formation or coadministered with ISCOMATRIX adjuvant, which is essentially the same structure but without the incorporated Ag (reviewed in Ref. 16). ISCOM vaccines induce efficient crosspresentation of tumor Ag by DC *via* facilitated Ag translocation into the cytosol.[17,18] A tumor vaccine using the ISCOMATRIX adjuvant has been evaluated in a placebo-controlled clinical trial in patients with resected NY-ESO-1 expressing tumors, displaying an excellent safety profile and broad cellular immune responses.[19] However, in another trial the same ISCOMATRIX vaccine failed to induce T-cell responses in patients with advanced tumors, indicative of tumor-induced immune suppression.[20]

In this study, we investigated the efficacy of an ISCOM-based tumor vaccine in an orthotopic model of pancreatic carcinoma. Pancreatic tumors induced a potent Ag-specific immunosuppression rendering the vaccine ineffective. Depletion of Treg or combining the vaccine with low-dose CpG was capable of breaking immunosuppression and induced effective CTL-mediated tumor cell killing leading to prolonged survival of animals with established pancreatic cancers.

Material and Methods

Cell culture media and reagents

Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS (Gibco BRL, Paisley, GB), 1% L-glutamine, 100 U/l penicillin, and 0.1 mg/ml streptomycin (all PAA, Linz, Austria). The OVA/ISCOM vaccine was generated by associating palmitified OVA (Sigma, A7461) into an ISCOM by formulation with ISCOPREP saponin, phospholipids and cholesterol, as previously described.[18] CpG oligonucleotide 1826 was purchased from Coley Pharmaceuticals. The H2-Kb restricted peptides $OVA_{257–264}$ (SIINFEKL), $TRP2_{181–188}$ (VYDFFVWL) and $p15E_{604–611}$ (KSPWFTTL) were purchased from Jerini Peptide Technologies (Berlin, Germany). CFSE was obtained from Invitrogen, CA.

Mice and immunizations

Six- to 12-week-old female C57BL/6 mice were obtained from Harlan–Winkelmann (Borchen, Germany). OT-1 mice were provided by Prof. Brocker (Department of Immunology, University of Munich). Animal experiments were approved by the local regulatory agencies. Mice were injected s.c. into the lower hind leg with the OVA/ISCOM vaccine in 20 μl of PBS (containing 0.3 μg OVA and 5 μg ISCOPREP saponin) at weekly intervals. Six micrograms of CpG were added if indicated. $CD8^+$ T cells or NK cells were depleted *via* i.p. injection of 500 μg of anti-CD8 mAB (clone YTS 169, BioXCell, West Lebanon) or 200 μg of anti-NK1.1 mAb (clone PK-136, BioXCell). Treg were depleted *via* i.p. injection of 250 μg of anti-CD25 mAb (clone PC61 BioXCell) 1 day prior to vaccination. Depletion efficacy was controlled by four-color FACS analysis.

Tumor induction

The Panc02 cell line (C57BL/6) is derived from a methylcholanthrene-induced pancreatic adenocarcinoma. Panc02 cells were transfected with the OVA plasmid (pAC-Neo-OVA, kindly provided by Prof. T. Brocker). The PancOVA cell line was maintained with 0.5 mg/ml G418. We experimentally induced pancreatic carcinomas by injection of 0.5×10^6 tumor cells s.c. into the flank. For the orthotopic model, the spleen was surgically mobilized and 2×10^5 PancOVA cells were injected into the pancreas. Therapeutic vaccination started between days 10–14, when tumors had formed palpable nodules of 5–10 mm^2. Tumor size was measured three times weekly and determined by the product of perpendicular diameters. Mice were euthanized when the tumor size exceeded 200 mm^2 or with the appearance of distress.

LN preparation and cytokine measurement

Vaccine draining LN were removed and processed to single cell suspensions by passing through a 70-μm cell strainer. Cells were counted and processed for phenotypic and functional analysis. For cytokine measurements, freshly isolated LN were shock frozen in liquid nitrogen, processed using a mortar and transferred into 30 μl of lysis buffer (BioRad, Germany). Samples were vortexed for 30 sec and centrifuged (15 min, 12,000*g*, 4°C). The cell pellet was discarded and the supernatant served for cytokine analysis. All samples were standardized using Bradford method. Murine ELISA kits for IL-12p70 and IFN-γ (OptEIA) were from BD Biosciences (San Diego, CA). Cytokine measurement and analysis of activation marker expression of lymph node leukocyte populations were done by processing vaccine site draining lymph nodes into single cell suspensions. For intracellular detection of IL-12 in DC, cells were surface stained for CD8α and

CD11c, fixed and permeabilized using a cytofix/cytoperm kit (BD Biosciences), stained for IL-12p40/70 and analyzed by FACS. Measurement of TGF-β serum levels was done with ELISA (eBiosciences, Frankfurt, Germany) according to the manufacturer's instruction. Serum was incubated with 1 N hydrochloric acid for 10 min, neutralized with 1 N sodium hydroxide and immediately used for analysis.

Monoclonal antibodies and FACS

CD3ε-APC (clone 145-2C11), CD4-PE (clone GK1.5), CD8α-PerCP (clone 53-6.7), CD11c-APC or CD11c-PE (clone HL3), CD19-PE (clone 1D3), CD86-FITC (clone GL1), NK1.1-PerCP (clone PK 136) and IL-12p40/70-APC (clone C15.6) were all from BD Biosciences; CD69-FITC (clone H1.2F3), from Caltag Laboratories, Carlsbad, CA. Treg cells were stained with the Mouse Regulatory Staining kit from eBiosciences (CD4-FITC, Foxp3-APC, CD25-PE). Samples were acquired on a FACSCalibur (Becton Dickinson). Data were analyzed using FlowJo software (version 7.2.1, Tree Star, OR).

Intracellular IFN-γ staining of Ag-specific T cells and MHC class I pentamer staining

For intracellular IFN-γ staining, peripheral blood was incubated with red blood cell lysis buffer (BD Pharm Lyse, BD) for 3 min. Lymphocytes were then stimulated with relevant peptides (1 μg/ml) for 1 hr at 37°C before 1 μg/ml Brefeldin A (Sigma-Aldrich) was added. After 3 hr, cells were surface stained for CD8, then fixed and permeabilized using a cytofix/cytoperm kit (BD Biosciences) and incubated with mAb against IFN-γ-FITC (clone XMG1.2, Caltag Laboratories). Pentamer staining was performed with H-2Kb $OVA_{257–264}$ R-PE pentamers (ProImmune, Oxford, UK) according to the manufacturer's instructions.

In vivo T-cell proliferation and cytotoxicity assay

Splenocytes from OT-1 mice were suspended at 5×10^7 cells/ml in PBS with 0.1% BSA containing 10 μM CFSE for 10 min at 37°C. A total of 2×10^6 CFSE-labeled OT-1 cells in 200 μl PBS were injected i.v. in tumor-bearing mice vaccinated once with OVA/ISCOM vaccine on the same day. After 60 hr, blood was collected and OT-1 T-cell proliferation was analyzed by FACS. For assessing CTL-mediated cytotoxicity splenocytes were labeled with 1 or 10 μM CFSE and pulsed with either $OVA_{257–264}$ peptide (1 μg/ml) or not. A total of 10^6 labeled cells of each population were mixed and injected i.v. into vaccinated or nonvaccinated mice. Blood was collected 16 hr later and analyzed by FACS. The proportion of target to control population was determined and percentage of killing was calculated as described.[13]

Immunohistology

Cryostat sections of tumors were air-dried and fixed in ice-cold acetone for 10 min. For analysis of tumor-infiltrating $CD8^+$ T cells sections were incubated with rat anti-mouse CD8 (BD Pharmingen) at a dilution of 1:50, followed by biotin-SP-conjugated donkey anti-rat IgG and Cy™2-conjugated streptavidin (Jackson ImmunoResearch Europe), both at 1:500. For detection of Treg sections were incubated with rat anti-mouse Foxp3 (eBioscience) and Syrian hamster anti-mouse CD3 (BD Pharmingen) at 1:50, followed by Cy™3-conjugated goat anti-Syrian hamster IgG (H+L) at 1:400 together with biotin-SP-conjugated donkey anti-rat IgG at 1:500 followed by Cy™2-conjugated streptavidin at 1:500 (all from Jackson ImmunoResearch Europe). Slides were covered with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA). Histological images were obtained using a Carl Zeiss Axiophot microscope equipped with a digital camera system (DMC 2 digital camera, Polaroid).

Statistics

We determined the statistical significance of differences by the two-tailed Student's *t*-test. For the analysis of tumor growth, we used the nonparametric Mann–Whitney *U* test to compare the mean values between two groups. Kaplan–Meier survival curves were analyzed using the Cox proportional hazards model. We performed statistical analysis with SPSS software. p values < 0.05 were considered significant.

Results

ISCOM vaccine fails to control growth of established pancreatic carcinoma due to tumor-induced immunosuppression via regulatory T cells

Mice were vaccinated on day 0 and boosted at day 7 with an ISCOM vaccine containing 0.3 μg of OVA protein. Seven days later mice were challenged s.c. with PancOVA tumor cells. All vaccinated animals were protected from tumor growth, whereas all control animals developed large tumors requiring euthanasia (tumor size > 200 mm^2) within 40 days (Fig. 1*a*). To assess the efficacy of the vaccine in a therapeutic setting, vaccination was initiated on day 10, when tumors had already formed palpable nodules. In this setting, vaccination influenced neither tumor growth nor survival (Fig. 1*b*).

We hypothesized that the discrepancy of clinical efficacy between prophylactic and therapeutic vaccination was due to tumor-induced immunosuppression. To assess whether tumors induce vaccination failure, we measured the frequency of Ag-specific CTL in the blood of tumor-bearing *versus* control mice after two vaccinations with OVA/ISCOM. Vaccination induced a high frequency of OVA-specific CTL in animals without tumors as well as with wild-type Panc02 tumors, as assessed by MHC class I pentamer staining and intracellular IFN-γ staining (ICS). In contrast, animals with PancOVA tumors had a significantly reduced OVA-specific CTL frequency (1.4% *vs.* 6.2% of $CD8^+$ T cells by ICS for IFN-γ, $p < 0.01$) (Fig. 1*c*). Thus, the tumor suppressed CTL induction in an Ag-specific manner.

It has been shown that tumors may impair the function of DC, which are key regulators of T-cell responses.[5] To test this hypothesis in our model we adoptively transferred CFSE-labeled $CD8^+$ T cells from TCR transgenic OT-1 mice, which recognize a H2-Kb restricted OVA epitope. Mice were then

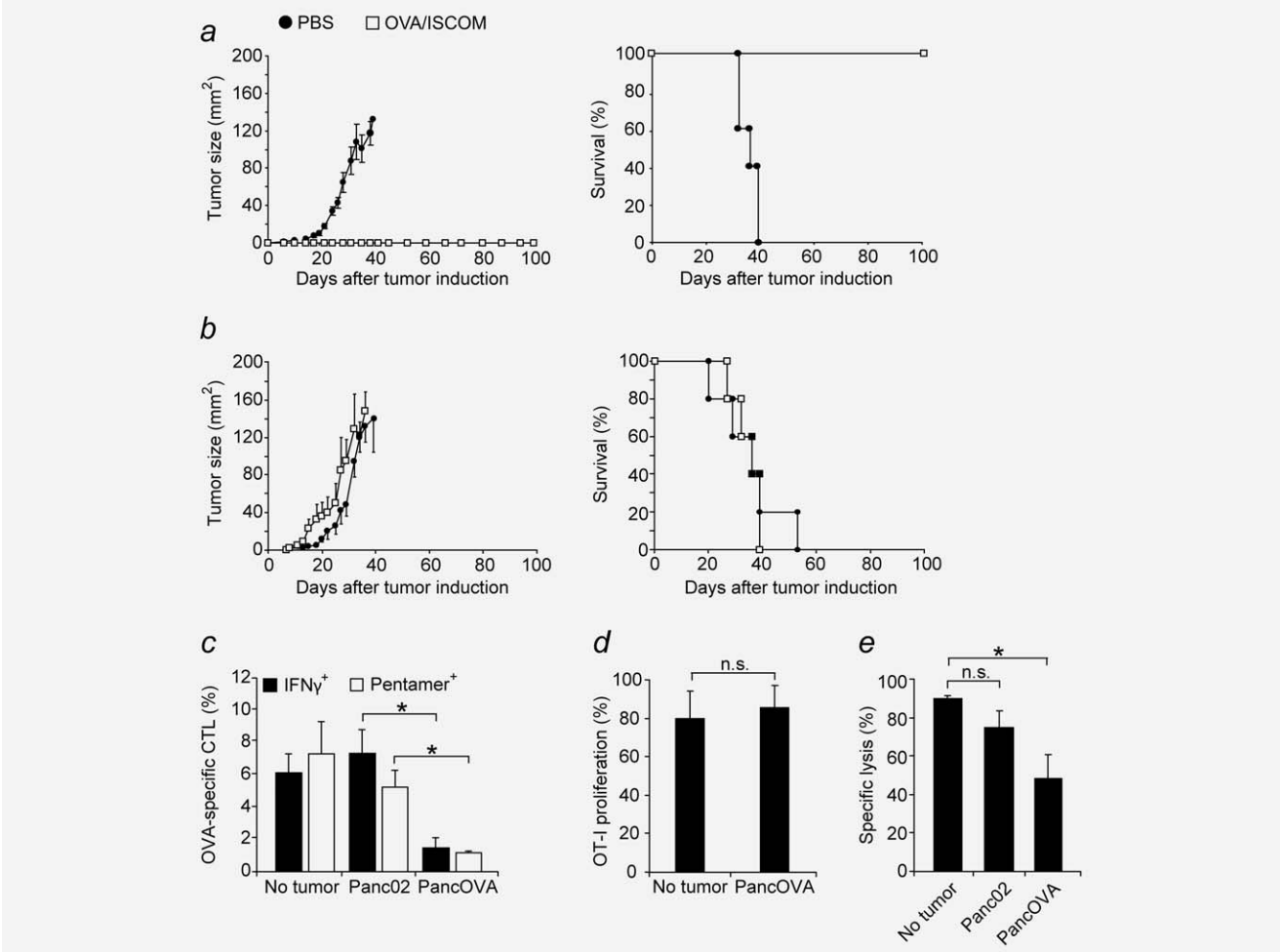

Figure 1. Therapeutic vaccination with an ISCOM vaccine is ineffective in pancreatic carcinoma. (*a*) Mice were injected twice with PBS or OVA/ISCOM at a weekly interval and challenged s.c. with OVA-expressing Panc02 (PancOVA) cells. (*b*) Mice with palpable PancOVA tumors were injected twice with OVA/ISCOM or PBS. Tumor growth (left graphs) and survival (right graphs) were monitored. Data of (*a*) and (*b*) are shown as mean tumor sizes with SEM and corresponding survival rates of six to eight mice per group from two independent experiments. (*c*) Mice with or without Panc02 or PancOVA tumors were treated with the OVA/ISCOM vaccine. Number of OVA-specific CTL in blood was determined 7 days after the second vaccination by *ex vivo* peptide stimulation and intracellular cytokine staining for IFN-γ of $CD8^+$ T cells or MHC class I pentamer staining. (*d*) Mice with or without tumors were vaccinated and CFSE-labeled OT-1 T cells were adoptively transferred *via* the tail vein. T-cell proliferation was determined by FACS analysis after 60 hr. (*e*) Mice with or without Panc02 or PancOVA tumors were vaccinated and CFSE-labeled, peptide-pulsed target cells were adoptively transferred. Target cell killing was determined by FACS analysis after 18 hr. Data in (*c–e*) are shown as mean with SEM of four to six mice per group. Representative data from three independent experiments are shown.

vaccinated and proliferation of CFSE-labeled $CD8^+$ T cells was assessed in peripheral blood. PancOVA tumors had no impact on OT-1 T-cell proliferation, indicating that $CD8^+$ T-cell stimulation by DC was unimpaired (Fig. 1*d*). To test killing function of CTL in tumor-bearing animals, we transferred CFSE-labeled peptide-pulsed target cells into vaccinated mice and assessed cytotoxicity *in vivo*. Target cell killing was reduced in mice with PancOVA tumors as compared to animals without tumors, correlating with reduced CTL frequencies in peripheral blood. In contrast, killing was not significantly different in mice with wild-type tumors (Fig. 1*e*).

TGF-β is a potent negative regulator of T-cell function and serum levels are increased in patients with pancreatic cancer.[7,21] Similarly, we found a significant increase of TGF-β serum levels in mice with pancreatic tumors (79 *vs.* 1515 pg/ml, $p < 0.01$) (Fig. 2*a*). Moreover, consistent with clinical observations,[7] we found an increased number of Treg in blood, lymph nodes and spleens, increasing from 12.5% in healthy to 18.6% of $CD4^+$ T cells ($p < 0.05$) in spleens of tumor-bearing mice on day 21 after tumor establishment (Fig. 2*b*). Particularly dense infiltrates of Treg were found in the tumors comprising 40% of total $CD4^+$ T cells (Fig. 2*b* and Supporting Information Fig. 1A). As Ag-specific suppression of T cells is a key feature of Treg,[22] we speculated that Treg play a central role in the immunosuppressive milieu created by pancreatic cancer. To assess the influence of Treg on the

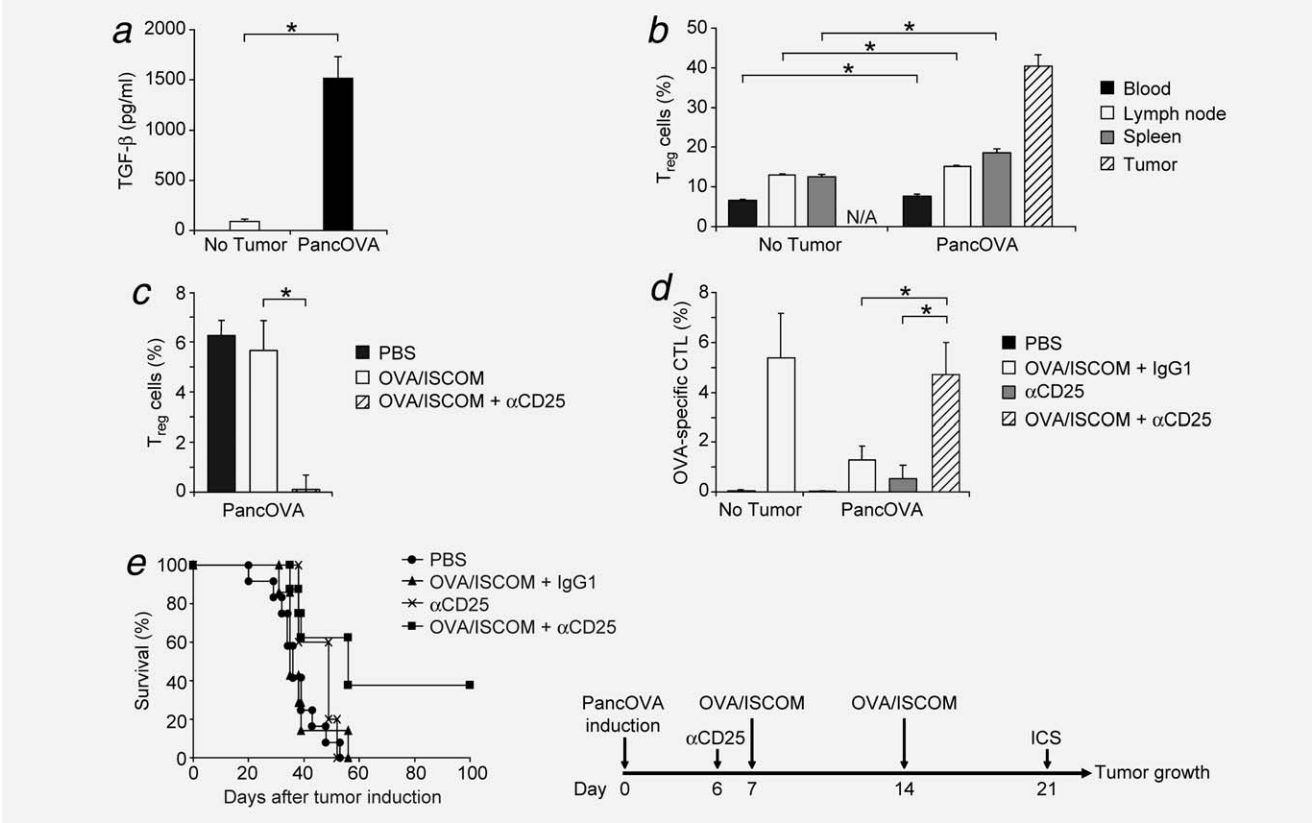

Figure 2. Pancreatic carcinoma mediates potent immunosuppression *via* regulatory T cells. (*a*) Serum TGF-β level of mice with or without PancOVA tumors was measured by ELISA. (*b*) Percentage of $CD25^+$ $Foxp3^+$ cells of $CD4^+$ T cells in blood, lymph nodes, spleen and tumors in control mice and mice with PancOVA tumors was analyzed by FACS. (*c*) Percentage of Treg cells in peripheral blood without and 6 days after i.p. injection of anti-CD25 mAb. (*d*) Mice with or without PancOVA tumors were vaccinated twice in weekly intervals and frequency of OVA-specific CTL was determined 7 days after second immunization by intracellular cytokine staining for IFN-γ. Where indicated mice were treated with anti-CD25 mAb or control mAb. (*e*) Influence of anti-CD25 mAb treatment on survival of mice with PancOVA tumors. Data are shown as mean with SEM of 5 (*a*–*c*) or 8 (*d*, *e*) mice per group. (*d*, *e*) Pooled data from two independent experiments.

vaccination failure in tumor-bearing animals we treated mice with anti-CD25 mAb one day prior to vaccination, which resulted in a long-lasting depletion of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg (Fig. 2*c*). Treg depletion restored CTL induction in mice with PancOVA tumors to levels seen in tumor-free animals and prolonged survival of vaccinated animals (Figs. 2*d* and 2*e*).

ISCOM vaccine combined with the TLR9 ligand CpG leads to a Th1 type immune response and activation of innate and adaptive immune cells in vaccine site draining lymph nodes

TLR agonists are potent inducers of immune activation and hold promise for tumor immunotherapy. The TLR9 agonist CpG is currently being evaluated in clinical trials, either alone or in combination with cytotoxic anticancer agents or tumor vaccines.[23] To characterize the effects of an ISCOM vaccine combined with CpG on the quality of the immune response, we isolated vaccine site draining lymph nodes for cytokine measurement (after 6 hr) and analysis of leukocyte populations (after 24 hr). The combined vaccine induced a potent Th1-polarized cytokine profile, characterized by high levels of IFN-γ and IL-12p70 (Fig. 3*a*). Intracellular cytokine staining of lymph node leukocyte populations showed IL-12p40/70 production by $CD11c^+CD8^+$ DC (Fig. 3*b*). No significant increase of IL-12 production above background level was observed in $CD11c^+CD8^+$ DC (data not shown). FACS analysis revealed activation of B cells, $CD4^+$ and $CD8^+$ T cells, NK cells, NKT cells and $CD8a^+$ DC in the combined treatment group (Fig. 3*c*). Of note, in contrast to high-dose CpG no significant change of activation marker expression was found in nondraining (collateral) lymph nodes or spleen (data not shown).

ISCOM vaccine combined with a TLR9 ligand leads to superior CTL priming and breaks tumor-induced immunosuppression

Next, we assessed how addition of CpG to the ISCOM vaccine influences CTL priming. A low-dose CpG regimen of 6 μg per mouse was chosen based on dose-response

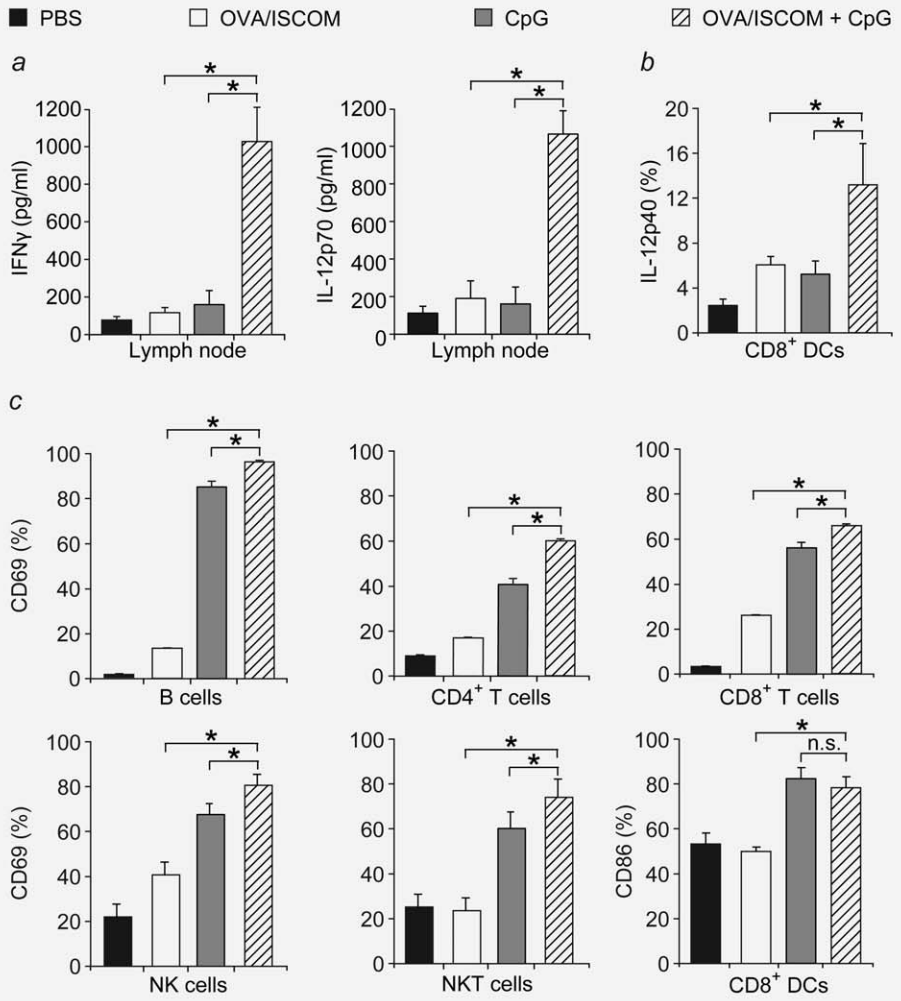

Figure 3. ISCOM vaccine combined with CpG leads to a Th1 type immune response and activation of innate and adaptive immune effector cells in vaccine site draining lymph nodes. (*a*) Mice with PancOVA tumors were vaccinated as indicated and the cytokine profile in the vaccine site draining lymph node lysate was analyzed after 6 hr by ELISA. (*b*) Intracellular cytokine staining for IL-12p40/70 of $CD11c^+CD8^+$ DC in vaccine site draining lymph nodes after 24 hr. (*c*) Activation marker expression of lymph node resident leukocyte populations such as $CD19^+$ B cells, $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells, $NK1.1^+$ NK cells, $NK1.1^+CD3^+$ NKT cells and $CD11c^+CD8^+$ DC. Data are mean with SEM of pooled lymph nodes of three mice per group. Data represent one experiment repeated twice.

experiments performed with tumor-free mice (data not shown). Combination with CpG significantly enhanced the frequency of OVA-specific CTL in vaccinated mice without tumors (8.5% without CpG *vs.* 25.4% with CpG, $p < 0.01$) (Fig. 4*a*). As shown before, CTL priming was significantly reduced in PancOVA-bearing animals (no tumor 8.5%, PancOVA 1.4%, $p < 0.01$). Interestingly, addition of CpG to the vaccine completely overturned tumor-induced immune suppression resulting in similar OVA-specific CTL frequencies as those observed in tumor-free animals (no tumor 25.4%, PancOVA 26.3%, $p = 0.89$) (Fig. 4*a*). Moreover, tumors of mice vaccinated with either the vaccine alone or combined with CpG showed an increase of total tumor infiltrating $CD8^+$ T cells (Fig. 4*b* and Supporting Information Fig. 1B). In addition, numbers of Treg in peripheral blood and infiltrating the tumor in the combined vaccine group were significantly reduced (Figs. 4*c* and 4*d*).

ISCOM vaccine combined with CpG leads to tumor regression and long-term survival of mice with pancreatic carcinoma in a $CD8^+$ T cell–dependent manner

To assess whether the combined vaccine can induce significant antitumor immunity, mice with palpable PancOVA tumors were injected with PBS, OVA/ISCOM, CpG or a combination of both. Neither CpG nor the vaccine alone influenced tumor growth or survival. In contrast, even large pancreatic tumors regressed in the combined vaccine group. Two of 10 mice showed delayed tumor growth and seven completely rejected their tumors and remained tumor-free for up to 100 days (Figs. 5*a* and 5*b*, left graph). To assess

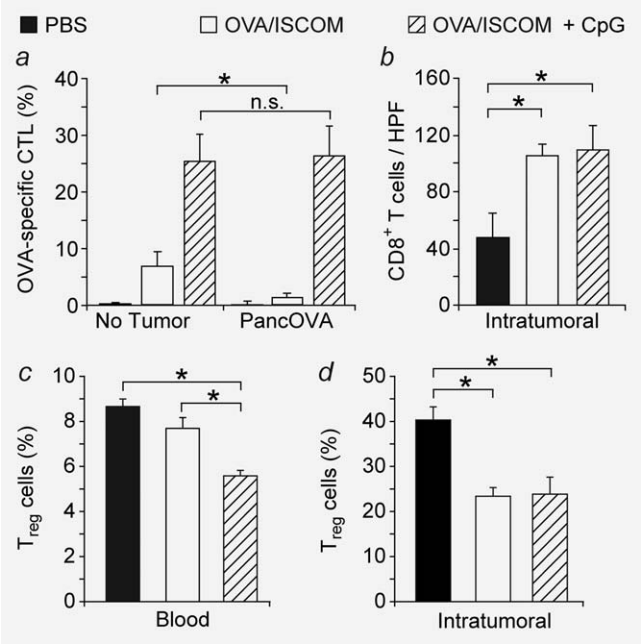

Figure 4. ISCOM vaccine combined with CpG leads to superior CTL induction, enhanced tumors infiltration with CTL and reduced numbers of regulatory T cells. (*a*) Mice with and without PancOVA tumors were vaccinated as indicated. Seven days after the second vaccination, frequency of OVA-specific, $CD8^+IFN\text{-}\gamma^+$ CTL in blood were analyzed. (*b*) Numbers of $CD8^+$ T cells within PancOVA tumors were analyzed by immunohistochemistry. (*c*, *d*) Numbers of $CD25^+$ $Foxp3^+$ cells of $CD4^+$ T cells in peripheral blood and within tumor stroma of vaccinated mice with PancOVA tumors. Data are shown as mean with SEM of four to six mice per group. One representative experiment of two is shown.

whether the combined vaccine is still effective in more advanced tumors, we treated mice around day 14 after tumor induction when tumors had an average size of 16 mm^2 (range: 9–24 mm^2). Tumors increased in size during the first week of treatment but started to regress a few days after the second vaccination. The combined vaccine induced complete tumor rejection in five of nine mice (max. tumor size 48 mm^2), demonstrating vaccine efficacy despite large tumor burden (Fig. 5*c*). As the biology of subcutaneous tumors may differ from orthotopic tumors, we next assessed this vaccination strategy in mice with surgically implanted pancreatic tumors. Vaccination was initiated 10 days after tumor implantation. All mice treated with PBS, OVA/ISCOM or CpG alone died of pancreatic tumors around day 40, whereas 80% of mice in the combined vaccine group survived an observation period of 100 days without evidence of tumor (Fig. 5*b*, right graph).

As TLR9 agonists induce both CTL and NK cell activation, we investigated which T-cell population is required for effective tumor killing. Prior to vaccination, we depleted $CD8^+$ T cells or NK cells by i.p. injection of mAb against CD8 or NK1.1, respectively. Mice treated with PBS or vaccine alone developed large pancreatic tumors, whereas all mice treated with the ISCOM vaccine combined with CpG rejected their tumors. Depletion of NK cells had no effect on tumor growth or survival. In contrast, depletion of $CD8^+$ T cells in the combined treatment group not only prevented vaccine-induced tumor regression, but even accelerated tumor progression, indicating a role of $CD8^+$ T cells in tumor control (Fig. 5*d*).

Long-term T-cell memory induction in mice with tumor regression and evidence of epitope spreading

Mice in the combined treatment group that had rejected tumors and remained tumor free for 100 days were assessed for T-cell memory induction by simultaneous re-challenge with PancOVA tumors on the left flank and wild-type Panc02 tumors on the right flank. As compared to age-matched control mice, all long-term survivors rejected PancOVA tumors, indicative of an efficient memory T-cell response (Fig. 6*a*, upper graph). Interestingly, 4 of 10 long-time survivors also rejected wild-type Panc02 tumors and 2 showed delayed tumor growth, indicative of vaccine Ag-unrelated antitumor immunity in 60% of mice (Fig. 6*a*, lower graph). A likely explanation for this observation is that killing of PancOVA tumors by vaccine-specific T cells triggered crosspriming of CTL recognizing other tumor-associated Ag, a phenomenon termed epitope spreading. To confirm this hypothesis, we analyzed the CTL response against p15E, a tumor-associated Ag derived from the MuLV env-protein expressed by Panc02 cells but not in healthy tissue. No p15E-specific CTL were detected in mice without tumor or in unvaccinated mice that were challenged with PancOVA tumors. In contrast, in long-term survivors we could detect p15E-specific CTL at a frequency of 1.0% in peripheral blood (Fig. 6*b*). This finding and the presence of OVA-specific CTL more than 3 months after vaccination underscore both T-cell memory induction and epitope spreading in animals of the combined treatment group.

Discussion

To develop effective tumor vaccines, it will be critical to identify strategies to break immunosuppressive mechanisms. To characterize the impact of pancreatic tumors on vaccine-induced CTL responses we used the aggressively growing Panc02 model. Orthotopic tumors were induced to imitate the clinical situation. We found several immunosuppressive phenomena, such as high serum levels of TGF-β, increased systemic numbers of Treg and dense Treg infiltrates in tumors. Our model thus reflects key immune phenomena found in human pancreatic cancer making it a valuable tool for preclinical research.[7,21]

Vaccination with an ISCOM vaccine protected all mice from subsequent tumor challenge. This finding is in line with other reports demonstrating efficiency of ISCOM vaccines in prophylactic tumor models.[24] In contrast, vaccination of mice with established tumors was ineffective, indicative of tumor-

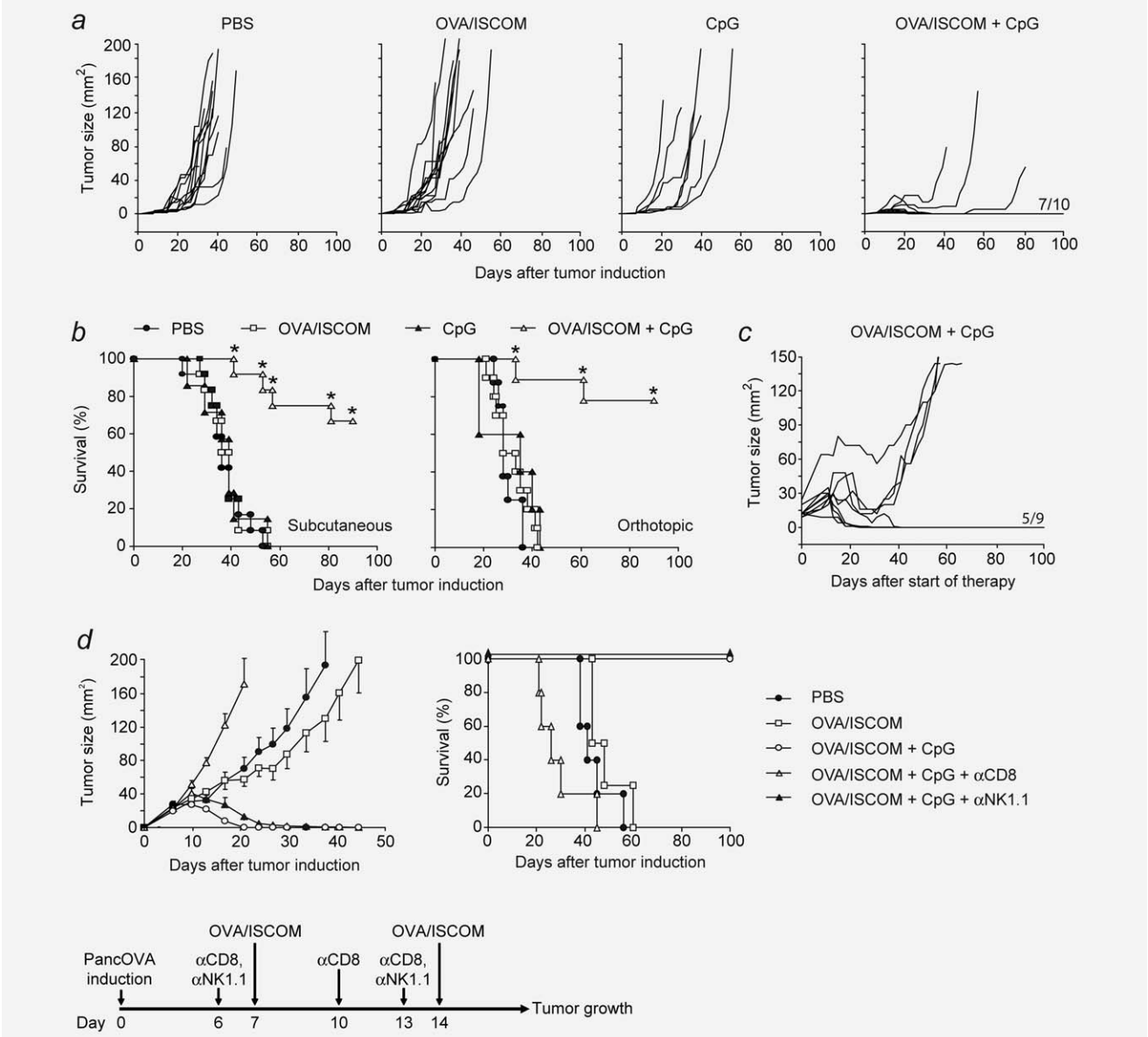

Figure 5. The combined vaccine leads to regression of subcutaneous and orthotopic pancreatic carcinoma and long-term survival mediated by $CD8^+$ T cells. (*a*) Mice with palpable PancOVA tumors were vaccinated twice as indicated at weekly intervals and tumor growth was monitored. (*b*) Survival rates in different treatment groups are depicted for subcutaneous and orthotopic tumors. Data sets of (*a*) and (*b*) represent 7–10 mice per group from two independent experiments. (*c*) Mice with advanced PancOVA tumors (tumor size: 9–24 mm^2) were vaccinated twice with OVA/ISCOM + CpG. Treatment was started around day 14 after tumor induction. Data represent nine mice from two independent experiments. (*d*) Mice with PancOVA tumors were vaccinated twice as indicated. $CD8^+$ T cells or NK cells were depleted by i.p. injection of CD8 or NK1.1 mAb prior to vaccination, as indicated. Data are shown as mean tumor size with SEM (left) and survival rates (right) of five mice per group.

induced immunosuppression. This was reflected by the finding that CTL induction in vaccinated mice with tumors was severely impaired. Our observation correlates well with two clinical studies in which an ISCOMATRIX vaccine was given to patients with either resected[19] or advanced malignant melanoma.[20] Broad-based T-cell responses were induced in patients with no measurable tumor burden in the first trial, but no T cells were induced in patients with advanced tumors in the second trial.

Regulation of T cells can occur at several levels: defective stimulation by DC, loss of T-effector cell function or inhibition by Treg (reviewed in Ref. [5]). In this study we did not find impaired CTL proliferation of transferred OT-I T cells in vaccinated tumor-bearing mice, arguing against defective

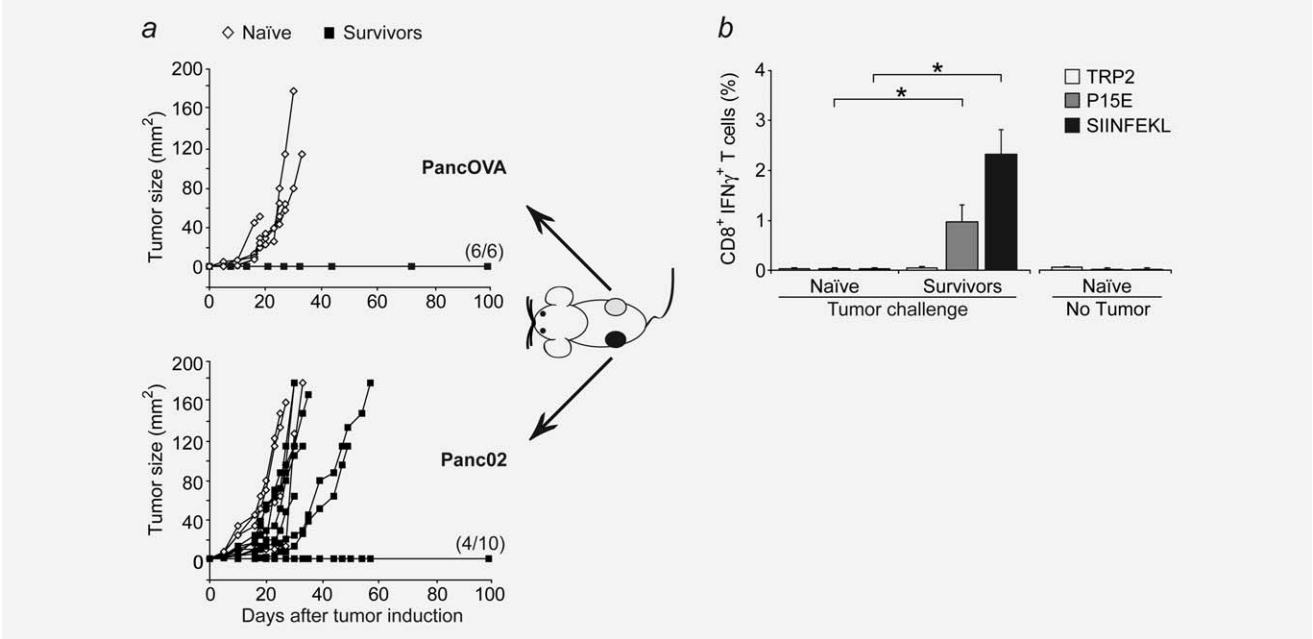

Figure 6. Mice with vaccine-induced tumor regression show evidence of long-term T-cell memory induction and epitope spreading. (*a*) Vaccinated mice that had rejected their tumors (survivors) and had no macroscopic evidence of residual tumor 3 months after primary tumor induction and age-matched controls (naïve) were (re-)challenged with PancOVA (left flank) and wild-type Panc02 (right flank) tumors. Data show individual tumor sizes of 6–10 mice per group. (*b*) Seven days after tumor re-induction, the frequency of Ag-specific, $CD8^+IFN\text{-}\gamma^+$ CTL was analyzed by *ex vivo* stimulation of blood leukocytes with H-2K^b restricted peptides from TRP2 (melanocyte differentiation Ag, unrelated), p15E (tumor-associated Ag, Panc02 and PancOVA) and OVA (SIINFEKL, PancOVA). Data are shown as mean with SEM of 6–10 (*a*) or 5 (*b*) mice per group from two experiments.

Ag presentation by DC. Furthermore, killing function of peptide-loaded target cells by CTL in peripheral blood was preserved, indicating that CTL were functional despite the tumor. High levels of TGF-β, a cytokine mediating the conversion from naïve $CD4^+$ T cells to Treg,[25] and increased systemic numbers of Treg point towards a critical role of this T-cell subset in immunosuppression by pancreatic carcinoma. In particular, the dense infiltrates of tumors with Treg maybe directly responsible for suppression of effector T cells upon arrival at the tumor site. A recent study showed that inhibition of CCR5-dependent homing of Treg to pancreatic cancer can modestly inhibit tumor growth, even in the absence of vaccination.[26] In our study, depletion of Treg with anti-CD25 mAb prior to vaccination restored CTL induction in mice with tumors and resulted in prolonged survival. Thus, Treg appear to play a critical role in immunosuppression induced by pancreatic tumors.

However, CD25 is not a specific Treg marker and other strategies to break Treg-mediated immunosuppression, such as DC activation *via* MyD88 signaling by TLR agonists, appear promising.[27] Several TLR agonists are under clinical investigation as vaccine adjuvants. Of these, TLR9 ligands are being evaluated in clinical phase II and III trials.[23] Hence, we combined the ISCOM vaccine with low-dose CpG and found a significant enhancement of CTL induction. This low-dose CpG regimen induced a potent Th1-type response with high levels of IL-12p70 and IFN-γ, as well as activation of innate and adaptive effector cells in vaccine site-draining LN. No systemic side effects such as splenomegaly and lymphoid follicle destruction were observed, which is associated with high-dose CpG application.[28] Importantly, combining the ISCOM vaccine with CpG was capable of breaking tumor-induced immune suppression leading to (*i*) efficient CTL induction despite tumor burden, (*ii*) recruitment of CTL to the tumor site, (*iii*) reduction of Treg in blood and tumors, (*iv*) efficient tumor cell killing mediated by CTL and (*v*) induction of T-cell memory.

Interestingly, the OVA/ISCOM vaccine alone was sufficient to induce an increase of $CD8^+$ T cells and a decrease of Treg in tumors. However, tumor growth was not affected without the addition of CpG to the vaccine, indicating that recruitment of T cells to the tumor site was not sufficient to break tumor-mediated immune suppression. A possible explanation for recruitment of $CD8^+$ T cells and a decrease of Treg at the tumor site is the immunostimulatory effect of ISCOMATRIX adjuvant. As shown in Figure 3, the ISCOM vaccine induced immune activation, as assessed by CD69 up-regulation on B cells, T cells and NK cells in draining LN. Furthermore, the vaccine induced high levels of IL-1β and IL-6 (data not shown). However, an additional stimulus, such as the TLR9 ligand CpG, was required for providing OVA-specific (and potentially other tumor Ag-specific) CTL with

tumor cell killing function. As a point of critique it can be argued that TLR9 ligands, which are highly active in mice, could be less active in humans due to differences in TLR9 expression in human and mouse DC populations.[23] In ongoing studies, we investigate combinations of ISCOM vaccines with ligands of other TLR (*i.e.*, TLR3, TLR4 and TLR7) finding enhanced CTL responses and antitumor efficacy (data not shown). Thus, TLR signaling seems to be a general mechanism for breaking tumor immunosuppression.[27]

A key element of adaptive immunity is the induction of memory, allowing the immune system to quickly and efficiently respond to subsequent Ag challenge. Mice that had rejected tumors in the combined treatment group were protected from subsequent tumor challenge, indicative of effective memory induction. Furthermore, 60% of mice developed an immune response against the wild-type tumor. This led us to hypothesize that successful therapy may induce activation of tumor-reactive CTL specific for vaccine-unrelated tumor Ag, *e.g.*, *via* crosspresentation of tumor Ag by DC in tumor-draining lymph nodes.[29] Indeed, in mice that had rejected the tumor we detected a high frequency of CTL against p15E, an Ag expressed by wild-type Panc02 cells.[13] Thus, ISCOM vaccines are capable of inducing epitope spreading, thereby diversifying antitumor CTL responses.

To our best knowledge, this is the first report of successful tumor vaccination leading to tumor eradication in the aggressive orthotopic Panc02 carcinoma model. Tumor vaccines may offer new treatment options for patients with pancreatic carcinoma and deserve further investigation. In this regard, survivin, a tumor Ag expressed by 90% of human pancreatic cancers,[30,31] is in preclinical evaluation in our laboratory as a recombinant, full length survivin protein ISCOM vaccine.

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Therapeutic Efficacy of Bifunctional siRNA Combining TGF-β1 Silencing with RIG-I Activation in Pancreatic Cancer

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Abstract

Deregulated TGF-β signaling in pancreatic cancer promotes tumor growth, invasion, metastasis, and a potent immunosuppressive network. A strategy for disrupting this tumor-promoting pathway is silencing TGF-β by siRNA. By introducing a triphosphate group at the 5′ end of siRNA (ppp-siRNA), gene silencing can be combined with immune activation via the cytosolic helicase retinoic acid-inducible gene I (RIG-I), a ubiquitously expressed receptor recognizing viral RNA. We validated RIG-I as a therapeutic target by showing that activation of RIG-I in pancreatic carcinoma cells induced IRF-3 phosphorylation, production of type I IFN, the chemokine CXCL10, as well as caspase-9–mediated tumor cell apoptosis. Next, we generated a bifunctional ppp-siRNA that combines RIG-I activation with gene silencing of TGF-β_1 (ppp-TGF-β) and studied its therapeutic efficacy in the orthotopic Panc02 mouse model of pancreatic cancer. Intravenous injection of ppp-TGF-β reduced systemic and tumor-associated TGF-β levels. In addition, it induced high levels of type I IFN and CXCL10 in serum and tumor tissue, systemic immune cell activation, and profound tumor cell apoptosis *in vivo*. Treatment of mice with established tumors with ppp-TGF-β significantly prolonged survival as compared with ppp-RNA or TGF-β siRNA alone. Furthermore, we observed the recruitment of activated $CD8^+$ T cells to the tumor and a reduced frequency of $CD11b^+$ $Gr\text{-}1^+$ myeloid cells. Therapeutic efficacy was dependent on $CD8^+$ T cells, whereas natural killer cells were dispensable. In conclusion, combing TGF-β gene silencing with RIG-I signaling confers potent antitumor efficacy against pancreatic cancer by breaking tumor-induced $CD8^+$ T cell suppression. *Cancer Res; 73(6); 1709–20. ©2013 AACR.*

Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death and is characterized by early metastasis and resistance to chemotherapy and irradiation. The identification of deregulated molecular pathways in pancreatic cancer and the development of novel targeted therapies had so far little impact on clinical outcome (1). Prognosis of patients with pancreatic cancer has remained extremely poor with a 5-year survival rate of less than 5%. A key event in tumor progression of pancreatic cancer is deregulation of TGF-β signaling (2). Under normal conditions, TGF-β maintains tissue homeostasis by controlling cellular proliferation, differentiation, survival, and cell adhesion. Deregulated TGF-β signaling allows tumors to usurp homeostatic effects for promoting tumor growth, invasion, metastasis, and tumor angiogenesis (2). Moreover, TGF-β has immunosuppressive effects such as inhibition of cytotoxic T cells and natural killer (NK) cells, induction of $FoxP3^+$ regulatory T cells, and shifting antigen-presenting cell function toward tolerance (3, 4). Both tumor cells and immune cells, such as regulatory T cells and myeloid-derived suppressor cells (MDSC), contribute to enhanced TGF-β production in patients with cancer. Elevated TGF-β levels in serum and tumors correlate with poor prognosis in patients with tumor (5, 6). Thus, TGF-β has generated interest as a target for novel anticancer agents. Anti-TGF-β compounds have shown efficacy in preclinical studies, and some of these have moved into clinical investigation for melanoma, brain tumors, colorectal, renal, and pancreatic cancer (7–11).

Tumor infiltration with T cells represents a positive prognostic factor for pancreatic carcinoma, indicating that immune surveillance may occur despite locally active immunosuppressive mechanisms (12). However, tumor-infiltrating T cells frequently lack effector function due to the hostile tumor microenvironment, which is enriched with immunosuppressive

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factors. Identifying targets and compounds for immune activation is thus of utmost importance for effective immunotherapy. In this respect, in addition to TGF-β inhibition, activation of innate immune effector mechanisms via pattern recognition receptors is a promising strategy for breaking tumor-mediated immunosuppression (13).

Eukaryotic cells have evolved pattern recognition receptors for the detection of viral nucleic acids to trigger appropriate antiviral immune responses. Four members of the toll-like receptor (TLR) family (TLR3, 7, 8, and 9) are located in the endosomal membrane predominantly in immune cells, whereas the retinoic acid-inducible gene I (RIG-I)-like helicases RIG-I and melanoma differentiation-associated gene-5 (MDA-5) are located in the cytosol of immune and nonimmune cells. Recent work suggests that RIG-I may represent a novel target for cancer immunotherapy (14, 15). RIG-I is a cytosolic sensor of viral RNA detecting a triphosphate group at the 5′ end generated by viral RNA polymerases (16, 17). Upon activation, RIG-I initiates a signaling cascade mediated by IRF-3, IRF-7, and NF-κB, leading to an antiviral response program characterized by the production of type I IFN (IFN-α and IFN-β) and other innate immune response genes, such as the chemokine CXCL10 (18). Moreover, RIG-I signaling induces apoptosis in susceptible cells. *In vitro* transcribed 5′-triphosphate RNA (ppp-RNA) can be applied as RIG-I ligand to trigger proapoptotic signaling via the intrinsic mitochondrial pathway (14, 15). Of note, RIG-I–mediated apoptosis occurs predominantly in tumor cells, as nonmalignant cells are protected from proapoptotic signaling via Bcl-x_L (14).

The novel ppp-siRNA strategy offers the advantage of combining RIG-I–mediated immune activation with RNAi-mediated gene silencing within a single molecule. In the study by Poeck and colleagues, a bifunctional ppp-siRNA silencing the antiapoptotic molecule Bcl-2 resulted in efficient tumor cell apoptosis in melanoma (15). To date, little is known about RIG-I expression in other cancer types. We hypothesized that dual targeting of immunosuppression via RIG-I activation and TGF-β silencing promotes efficacy against pancreatic cancer. We studied RIG-I expression and signaling in human pancreatic carcinoma cell lines. In addition, the therapeutic efficacy of a bifunctional ppp-siRNA combining ppp-RNA–mediated RIG-I signaling with siRNA-mediated TGF-β silencing was assessed in the aggressive Panc02 mouse model of pancreatic carcinoma.

Materials and Methods

Cell lines and cytokine stimulation

The Panc02 cell line was established from a tumor that was induced by 3-methylcholanthren in the pancreas of *C57BL/6* mice and was a kind gift of Prof. Christiane Bruns (Chirurgische Klinik, LMU Munich, Bavaria, Germany; ref. 19). The human pancreatic adenocarcinoma cell lines PANC-1, MIAPaCa-2, and BxPC-3 cell lines were obtained from American Type Culture Collection (ATCC) and were used within 6 months after resuscitation (ATCC). PaTu8988t cell line was obtained from the German Collection of Cell Lines (DSMZ). IMIM-PC1 was kindly provided by Prof. Patrick Michl (Department of Gastroenterology and Endocrinology, University of Marburg, Marburg, Germany; ref. 20). Cancer cell lines were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum (Gibco BRL), 2 mmol/L L-glutamine, 100 U/L penicillin, and 0.1 mg/mL streptomycin (PAA). Each cell line was routinely tested for mycoplasma contamination by MycoAlert Mycoplasma Detection Kit (LONZA). IFN-α was used at concentrations of 1,000 IU/mL for murine and 100 IU/mL for human cell lines (Miltenyi).

siRNAs and transfection

siRNAs against TGF-β_1, RIG-I, Puma, and Noxa were designed according to published guidelines and were purchased from Eurofins MWG Operon (21). The matching 5′-triphosphate–modified siRNA was transcribed using the correlating DNA template that contained the T7 RNA polymerase promoter sequence. *In vitro* transcription of ppp-RNA was done using the MEGAshortscript T7 Kit (Ambion) according to the manufacturer's protocol (14, 15). All sequences can be found in Supplementary Table S1. Tumor cells were transfected with siRNA in OptiMEM (Gibco, BRL) using Lipofectamine 2000 (Invitrogen). For *in vivo* administration, 50 μg of RNA was complexed with *in vivo*-JetPEI (Peqlab) at an N/P ratio of 6 in 5% glucose solution for tail vein injection.

Mice, tumor engraftment, and therapy

Six-week-old female *C57BL/6* mice were obtained from Harlan-Winkelmann, *Trif*$^{-/-}$ and *Tlr7*$^{-/-}$ mice in a F6 C57BL/6 background originated from S. Akira (Department of Host Defense, Osaka University, Japan) and B. Beutler (Center for the Genetics of Host Defense, University of Texas Southwestern Medical Center, Dallas, TX; refs. 22, 23). Experiments were carried out according to ethical guidelines approved by the local government. Orthotopic tumors were induced by surgical implantation of 2×10^5 Panc02 cells into the pancreas as described (24). Therapy started on day 10, when sacrificed control mice showed visible tumor nodules (5–8 mm diameter), and was administered twice weekly over 3 weeks. Serum was obtained 6 hours after the first RNA injection for cytokine analysis, and 48 hours after second and fifth injections for TGF-β measurement. Survival of mice was monitored daily. Distressed mice were sacrificed. Where indicated, $CD8^+$ T cells or NK cells were depleted one day before RNA treatment with 500 μg of anti-CD8 monoclonal antibody (mAb; clone YTS 169.4) or anti-NK1.1 mAb (clone PK136; BioXCell). Depletion efficacy was assessed by fluorescence-activated cell sorting (FACS) analysis of peripheral blood and was more than 98% after 24 hours.

Protein preparation and Western blot analysis

Tumor tissue was snap frozen in liquid nitrogen and homogenized using mortar and pestle under constant liquid nitrogen cooling. Cells were lysed (Bio-Plex Cell Lysis Kit, Bio-Rad) and protein concentrations of supernatants were analyzed by Bradford assay (Bio-Rad) and adjusted for whole protein concentration. For Western blotting, cells were lysed in Laemmli buffer and boiled for 10 minutes. Cell lysates were separated using a 10% SDS-PAGE. RIG-I was detected with anti-RIG-I mAb (clone ALME-1, ENZO Life Sciences GmbH)

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followed by horseradish peroxidase-coupled secondary antibody (Santa Cruz Biotechnology). Anti-pIRF-3 mAb was from New England BioLabs. Blots were visualized using enhanced chemiluminescence substrate (GE Healthcare).

ELISA

Cytokine levels in supernatants, serum, or tumor lysates were quantified by ELISA for IFN-α and IFN-β (PBL Interferon Source), CXCL10 (R&D Systems), TGF-β (eBiosciences), and TNF-α (BD Biosciences).

Flow cytometry

Lymphocyte activation in spleens was assessed 12 hours after *in vivo* administration of RNA. Spleens were removed and processed into a single cell suspension for staining. Following antibodies were used: anti-CD3ε (clone 145-2C11), anti-CD4 (clone RM4-5), anti-CD8α (clone 53-6.7), anti-CD19 (clone 1D3), anti-NK1.1 (clone NKR-P1B, NKR-P1C, all BD Biosciences), and anti-CD69 (clone H1.2F3, Caltag Laboratories). MDSCs were analyzed with: anti-CD11b (clone M1/70), anti-Gr-1 (clone RB6-8C5), anti-Ly6G (clone 1A8), anti-Ly6C (clone AI-21), anti-CD11c (clone HL3), anti-CD80 (clone 16-10A1), and anti-Sca-1 (clone D7). For the characterization of MDSC subpopulations, Gr-1$^+$CD11b$^+$ cells were further subdivided by differential expression of the Ly6G. Briefly, Gr-1$^+$CD11b$^+$Ly-6G^{hi} cells were defined as polymorphonuclear (PMN)-MDSCs, whereas Gr-1$^+$CD11b$^+$Ly-6G$^{lo/neg}$ cells were classified as the monocytic MDSCs. For MHC-I staining, anti-human HLA-A, B, or C (clone G46-2.6) or anti-mouse H-2D^b (clone KH95, both BD Biosciences) was used. Samples were acquired on a FACSCanto II (BD) and data analyzed using FlowJo software (Version 8.5.3, Tree Star Inc.).

Detection of apoptosis

Apoptosis was determined using Annexin V-APC (Invitrogen) and propidium iodide staining by flow cytometry. Activation of caspase-9 in tumor cells was analyzed by flow cytometry using the caspase-9 and caspase-3/7 FLICA Kits (Immunochemistry, Biomol). Caspase-9 Western blot analysis was done using mouse anti-caspase-9 mAb detecting both the proform and active form (New England Biolabs GmbH). Activated caspase-9 in tumor lysates was quantified by colorimetric analysis using the Caspase-9 Activity Detection Kit (Abcam). TUNEL staining was conducted using the In Situ Cell Death Detection Kit (Roche) and mounted with Vectashield w/DAPI (Vector Laboratories) for nuclei visualization. Stained tissues were visualized by confocal fluorescence microscopy (Leica TCS SP5).

Quantitative real-time PCR

Total mRNA was isolated using the RNeasy Kit (Qiagen). cDNA was transcribed using Protoscript First Strand DNA Synthesis Kit (New England BioLabs). Quantitative real-time PCR (qRT-PCR) was conducted using the LC 480 Probes Master Kit and Light Cycler 480 instrument (Roche Diagnostics). Primers were designed with the Universal Probes library (Roche). The copy numbers of each sample were correlated to hypoxanthine phosphoribosyltransferase.

Histology

Hematoxylin and eosin (H&E) staining was conducted according to common protocols. For immunohistology, paraffin-embedded specimens were cut at 3 μm. After deparaffinization and rehydration, heat pretreatment was done with ProTaqs V Antigen Enhancer (Quartett, Immunodiagnostika & Biotechnologie GmbH). The staining was conducted using mouse anti-RIG-I mAb (Enzo Life Sciences AG). Detection was accomplished by Real Detection System APAAP (Dako) and counter stained with haematoxylin Gill's Formula (Vector Laboratories). Lymphocyte infiltrates in Panc02 tumors were analyzed with mAb anti-CD3 (clone 17A2) and anti-CD8 (clone 53-6.7, all BD Pharmingen). Images were obtained by fluorescence microscopy (Axiovert 2000, Carl Zeiss) and processed with Adobe Photoshop CS4 for adjustment of contrast and size.

Statistical analysis

Data present mean + SD (*in vitro* data) or SEM (*in vivo* data). Significant differences were analyzed using 2-tailed Student *t* test. Multiple comparisons were analyzed by one-way ANOVA including Bonferroni correction. Survival curves were analyzed with Mantel–Cox test. Statistical analysis was conducted using GraphPad Prism software (version 5.0a); P values < 0.05 were considered significant.

Results

Pancreatic carcinoma cells express functional RIG-I

RIG-I expression was studied in human PanIN lesions, primary pancreatic adenocarcinomas, and metastases by immunohistochemistry. We found strong cytosolic staining for RIG-I in premalignant lesions and in tumor cells in 10 of 10 specimens (Supplementary Fig. S1). We also assessed RIG-I expression by qRT-PCR and Western blot analysis in various human pancreatic cancer cell lines, including PANC-1, PaTu8988t, MIAPaCa-2, IMIM-PC-1, and BxPC-3 that were cultured in the absence or presence of IFN-α. All human pancreatic cancer cell lines tested expressed basal levels of RIG-I protein that were upregulated upon IFN-α treatment (Fig. 1A). We next assessed RIG-I signaling in pancreatic cancer cells in response to treatment with the RIG-I ligand ppp-RNA. We observed phosphorylation of the nuclear transcription factor IRF-3 in all cell lines (Fig. 1B). Furthermore, RIG-I stimulation induced the secretion of CXCL10 (IP-10) and IFN-β (Fig. 1C and D). The cell line MIAPaCa-2 lacked IFN-β production due to a deletion of the *IFN-β* gene (25) but showed IRF-3 phosphorylation and secreted CXCL10, indicative of intact RIG-I signaling. In addition, FACS analysis revealed upregulation of MHC-I surface expression in all cell lines (Fig. 1E). To confirm that these effects were mediated by RIG-I, we silenced RIG-I in PANC-1 cells with siRNA before ppp-RNA stimulation. Phosphorylation of IRF-3, CXCL10 secretion, and MHC-I expression were significantly reduced in RIG-I–silenced cells (Fig. 1F and G). Together, these data show that human pancreatic cancer cells express functional RIG-I and validate RIG-I as a potential therapeutic target.

We next investigated RIG-I expression in the murine pancreatic carcinoma cell line Panc02, which forms highly aggressive tumors in *C57BL/6* mice. RIG-I expression was low in

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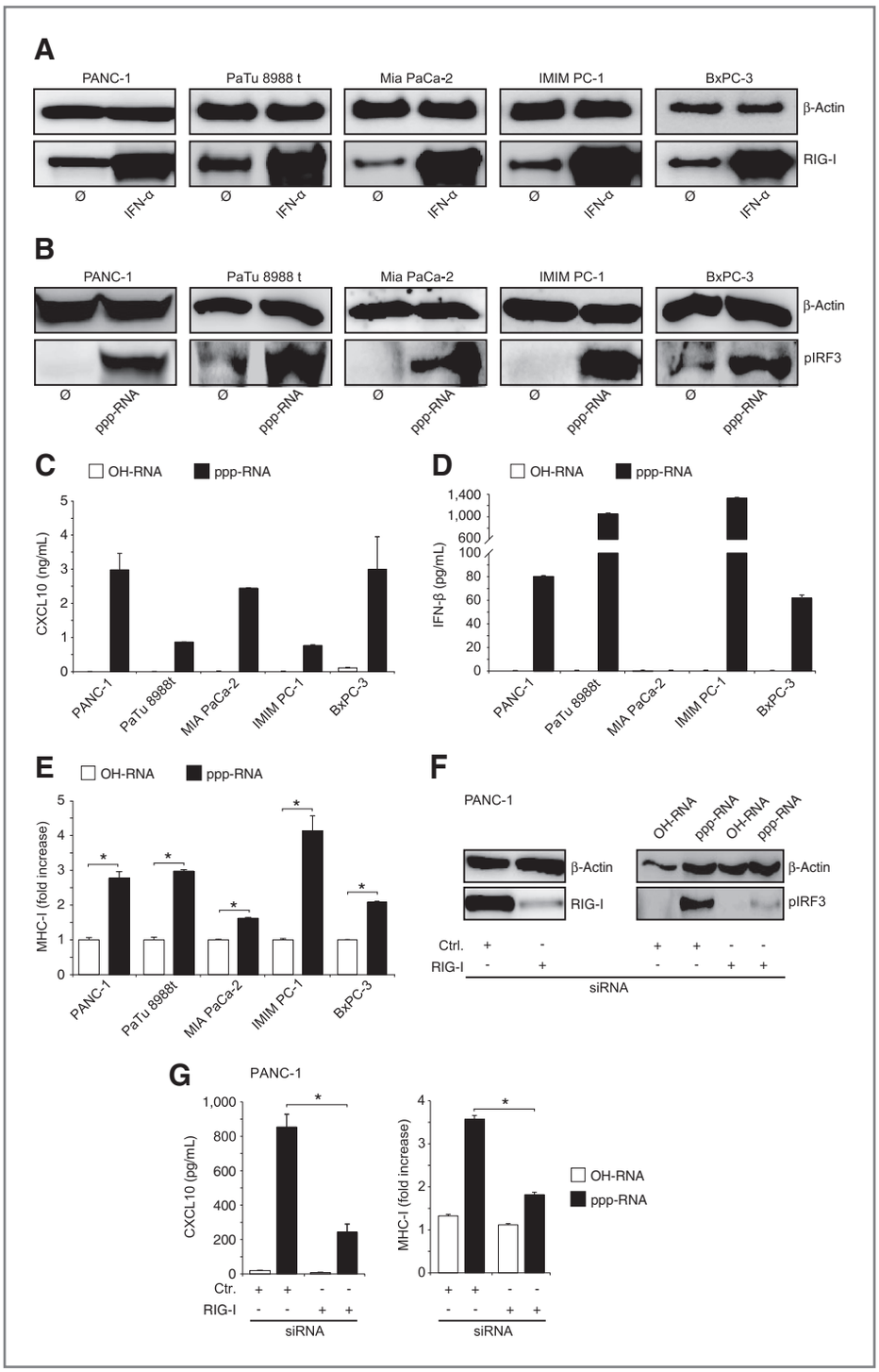

Figure 1. Human pancreatic cancer cells express functional RIG-I. A, different human pancreatic cancer cell lines were cultured in the absence or presence of 100 IU/mL IFN-α or 1 μg/mL ppp-RNA for 12 hours. Expression of RIG-I protein was determined by Western blot analysis. B, tumor cells were stimulated with ppp-RNA (1 μg/mL) for 2 hours and phosphorylated IRF-3 (pIRF-3) was assessed by Western blot analysis. C–E, pancreatic cancer cells were treated with 1 μg/mL OH-RNA or ppp-RNA for 24 hours. Release of CXCL10 (C) and IFN-β (D) was measured by ELISA. E, MHC-I surface expression was measured by flow cytometry. MHC-I expression levels are shown as fold increase compared with cells treated with control OH-RNA. F, PANC-1 cells were incubated with irrelevant (Ctrl.) or RIG-I–specific siRNA for 24 hours and subsequently stimulated with OH-RNA or ppp-RNA (1 μg/mL) for 2 hours. pIRF-3 was assessed by Western blot analysis. Efficiency of RIG-I silencing was assessed by Western blot analysis. G, PANC-1 cells were incubated with irrelevant (Ctr.) or RIG-I–specific siRNA for 24 hours and subsequently stimulated with 1 μg/mL OH-RNA or ppp-RNA. CXCL10 levels and MHC-I expression were analyzed after an additional 24 hours. Mean + SD from triplicates of 1 of 3 and representative images of 3 independent experiments are shown. *, $P < 0.05$.

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Panc02 cells under basal conditions but strongly upregulated upon IFN-α treatment (Fig. 2A). As observed with human cell lines, treatment with ppp-RNA resulted in the phosphorylation of IRF-3 as well as secretion of CXCL10 and IFN-β and upregulation of MHC-I expression (Fig. 2B and C). Again, RIG-I silencing confirmed the critical role of RIG-I signaling for these effects (Fig. 2D). Thus, the Panc02 model enabled us to study the therapeutic potential of ppp-RNAs *in vivo* in immunocompetent mice.

RIG-I signaling induces apoptosis in pancreatic carcinoma cells

In previous studies, RIG-I signaling was shown to induce apoptosis in melanoma cells via the intrinsic, caspase-9–dependent pathway involving upregulation of the proapoptotic BH3-only proteins Noxa and Puma (14, 15). We next assessed whether this proapoptotic pathway is also active in pancreatic cancer cells. Treatment with ppp-RNA strongly induced apoptosis in both human and murine pancreatic carcinoma cells as determined by PARP cleavage (data not shown) and Annexin V binding, which was strongly reduced in RIG-I–silenced tumor cells (Fig. 3A and B). In line with activation of the intrinsic apoptosis pathway, we observed activation of caspase-9 and caspases 3/7 (Fig. 3C and D). Moreover, ppp-RNA induced upregulation of Puma and Noxa in pancreatic cancer cells (Fig. 3E). Interestingly, siRNA-mediated silencing of Puma significantly inhibited apoptosis induction. Together, these results show that pancreatic cancer cells are sensitive to proapoptotic RIG-I signaling and confirm a role of proapoptotic BH3-only proteins in RIG-I–induced apoptosis.

Bifunctional ppp-TGF-β combines TGF-β gene silencing with RIG-I activation *in vitro*

To assess whether RIG-I activation and RNAi-mediated silencing of TGF-β can be combined in a single molecule, we designed a siRNA-targeting TGF-β_1 and the corresponding ppp-siRNA by *in vitro* transcription using a DNA template of the same sequence containing the T7 RNA polymerase promoter sequence (16). Unmodified siRNA carrying a free 5′-OH group (OH-TGF-β) and ppp-modified siRNA (ppp-TGF-β) reduced TGF-β to a similar extent in Panc02 cells on mRNA and protein levels (Fig. 4A). Thus, silencing activity was not impeded by the ppp modification. Moreover, ppp-TGF-β induced upregulation of CXCL10, IFN-β, and MHC-I as well

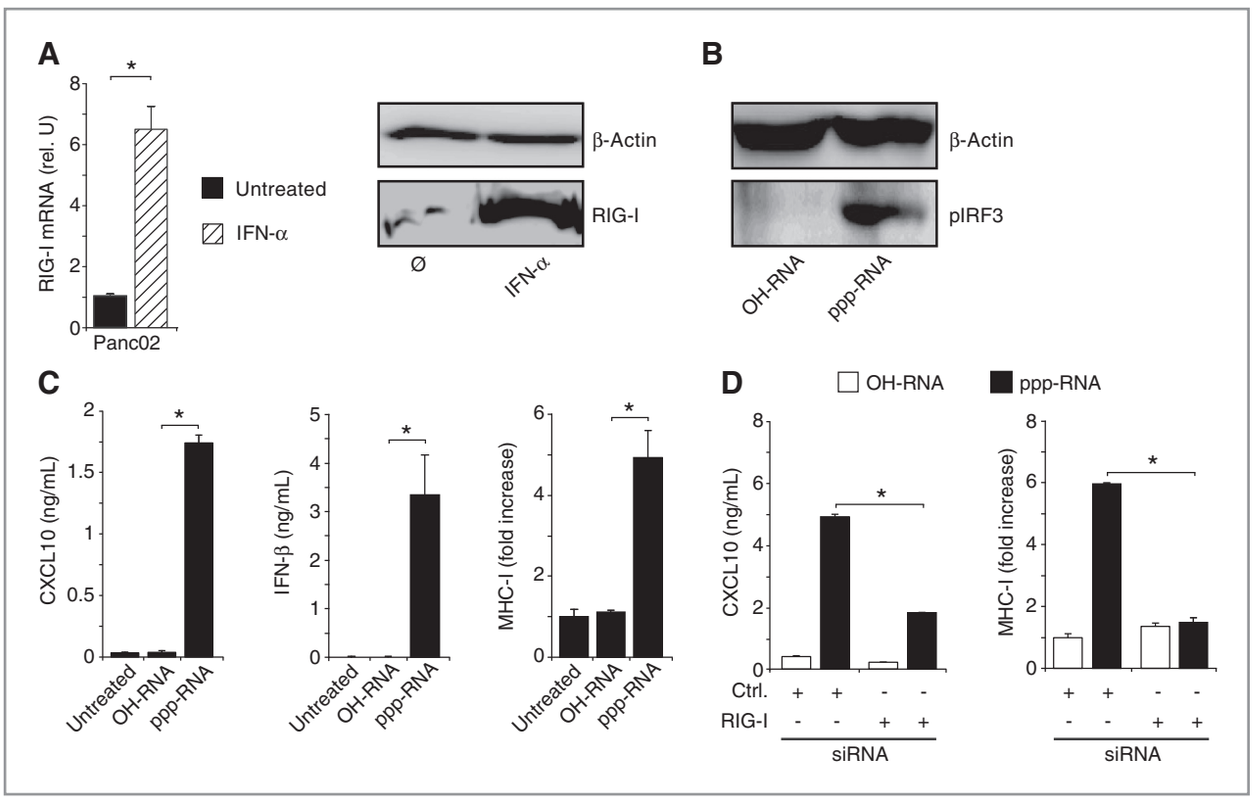

Figure 2. Murine Panc02 pancreatic carcinoma cells express functional RIG-I. A, Panc02 cells were cultured in the absence or presence of 1,000 IU/mL IFN-α for 12 hours. Expression of RIG-I was determined by qRT-PCR and Western blot analysis. B, phosphorylation of IRF-3 was assessed by Western blot analysis after treatment with OH-RNA or ppp-RNA (1 μg/mL) for 2 hours. C, Panc02 cells were stimulated with 1 μg/mL OH-RNA, ppp-RNA, or left untreated for 24 hours. CXCL10 and IFN-β secretion were analyzed by ELISA. MHC-I surface expression was measured by flow cytometry and was expressed as fold increase compared with untreated cells. D, Panc02 cells were incubated with irrelevant (Ctrl.) or RIG-I–specific siRNA for 24 hours and subsequently stimulated with OH-RNA or ppp-RNA. CXCL10 levels in supernatants were measured by ELISA and MHC-I expression by flow cytometry. Mean + SD from triplicates of 1 of 3 independent experiments. *, $P < 0.05$.

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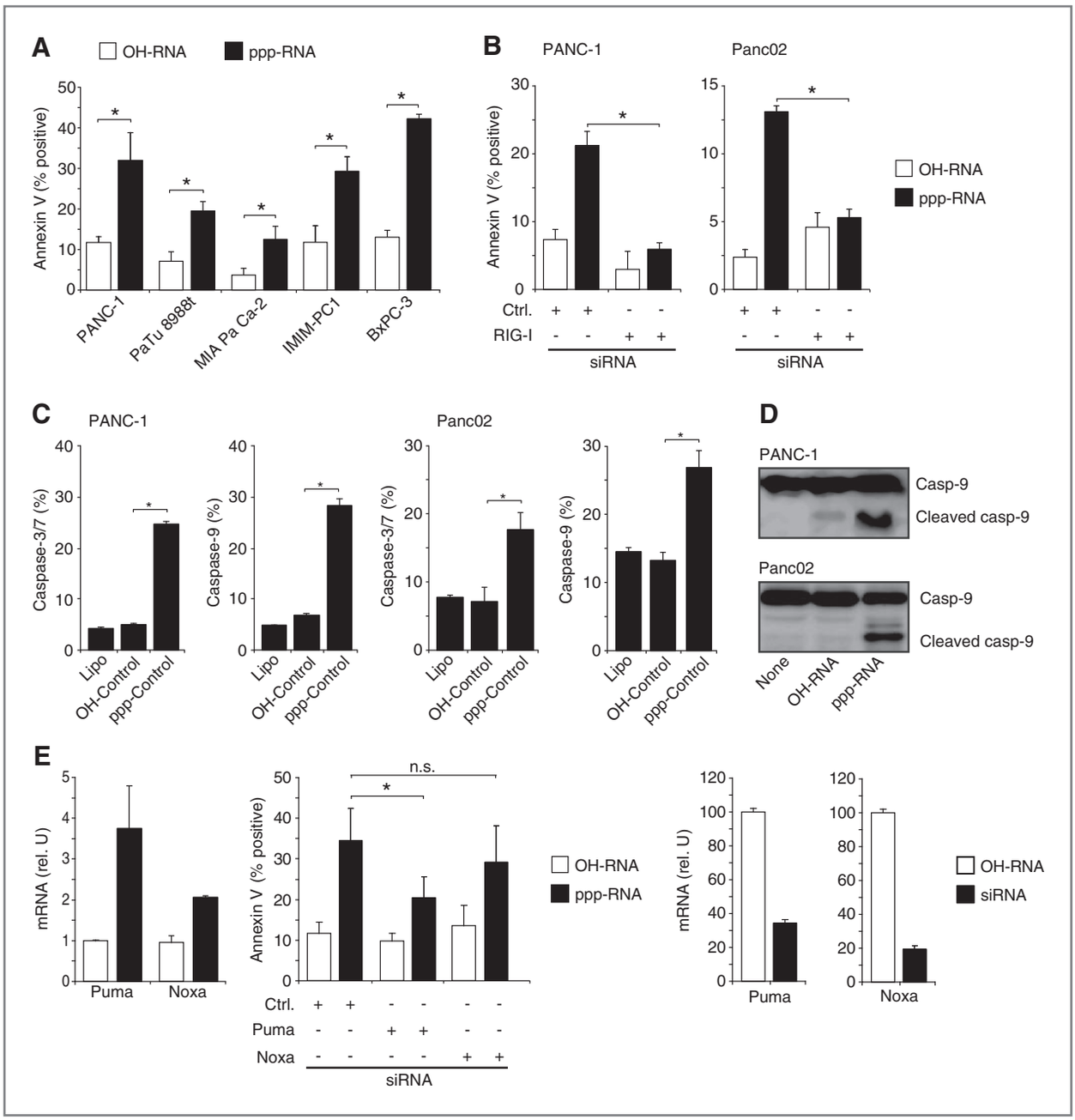

Figure 3. ppp-RNA induces apoptosis in murine and human pancreatic cancer cells. A, different human pancreatic cancer cell lines were treated with OH-RNA or ppp-RNA (2 μg/mL each) for 48 hours. Induction of apoptosis was measured via Annexin V/propidium iodide staining by flow cytometry. B, PANC-1 and Panc02 cells were incubated with irrelevant (Ctrl.) or RIG-I–specific siRNA for 24 hours and subsequently stimulated with OH-RNA or ppp-RNA. After additional 48 hours, apoptosis was studied by flow cytometry. C, PANC-1 and Panc02 cells were treated with Lipofectamine, OH-RNAs, or ppp-RNAs for 48 hours as indicated and activation of caspase-3/7 and caspase-9 was measured by flow cytometry using corresponding FLICA kits. D, activation of caspase-9 in PANC-1 and Panc02 cells was assessed by Western blot analysis. E, expression levels of Puma and Noxa in Panc02 cells in response to ppp-RNA was assessed by qRT-PCR. Effect of Puma or Noxa silencing on apoptosis induction by ppp-RNA is shown. Representative data of 3 independent experiments are shown. Bars represent mean + SD from triplicates. *, $P < 0.05$.

as apoptosis of Panc02 cells to a similar extent as control ppp-RNA (Fig. 4B–D). Of note, silencing of TGF-β with OH-TGF-β by itself had no influence on the viability of Panc02 cells. Similar results were obtained with a ppp-siRNA–targeting TGF-β in human PANC-1 cells (Supplementary Fig. S2). Thus, ppp-TGF-β effectively combines RNAi-mediated TGF-β silencing with ppp-RNA–mediated RIG-I activation in pancreatic cancer cells.

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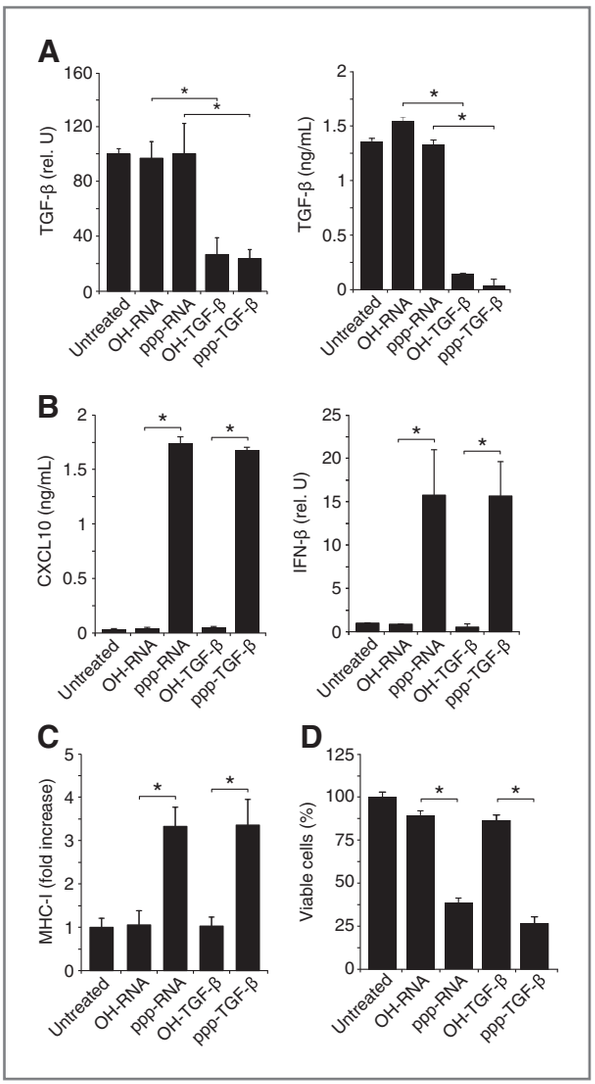

Figure 4. Bifunctional ppp-siRNA directed against TGF-β_1 combines gene silencing with RIG-I signaling. Panc02 cells were incubated with different RNAs (0.5 μg/mL) with or without silencing activity against TGF-β for 24 hours or left untreated. A, TGF-β levels were analyzed by qRT-PCR and ELISA. B, CXCL10 was measured in supernatants by ELISA and IFN-β expression by qRT-PCR. MHC-I expression (C) and viability (D) were assessed by flow cytometry. Mean + SD from triplicates of 1 of 3 independent experiments. *, $P < 0.05$.

Treatment of tumor-bearing mice with ppp-TGF-β leads to immune activation and TGF-β silencing *in vivo*

Next, we examined the immunostimulatory activity of ppp-TGF-β in the Panc02 pancreatic cancer model. Ten days after orthotopic tumor induction, mice were treated intravenously with RNAs, and cytokine production was measured in serum. Injection of ppp-RNA and ppp-TGF-β induced high serum levels of CXCL10, IFN-α, and moderate levels of TNF-α (Fig. 5A). In addition, we observed a potent systemic immune activation as evidenced by a strong upregulation of CD69 expression on B cells, $CD4^+$, and $CD8^+$ T cells, as well as NK and natural killer T cells (NKT; $NK1.1^+/CD3^+$) (Fig. 5B). Of note, intermediate levels of immune activation were also observed for siRNA against TGF-β. However, these were strongly reduced in *TLR7*$^{-/-}$ mice, indicative of a previously described off-target effect of unmodified siRNA (Supplementary Fig. S3; 26, 27). In contrast, immune activation in response to ppp-TGF-β treatment was not affected in mice lacking either TLR7 or TRIF (TLR3 signaling).

A hallmark of pancreatic cancer is the expansion of MDSCs that effectively suppresses $CD8^+$ T cell responses (28–30). Both, TGF-β blockade and type I IFN have been reported to reduce the suppressive function of MDSC (31, 32). We therefore investigated the effect of ppp-TGF-β treatment on MDSC in spleens of mice with Panc02 tumors. Strikingly, we observed a reduction in $CD11b^+$ $Gr\text{-}1^+$ MDSC numbers by 50%. This reduction was due to increased apoptosis of MDSC, as shown by enhanced caspase-9 activation (Fig. 5C). A similar trend was observed for OH-TGF-β, but lacked significance. Furthermore, ppp-TGF-β induced a shift from $Ly6G^+$ PMN-MDSC to $Ly6C^+$ monocytic MDSC and upregulation of CD11c, CD80, and Sca-1 expression (Supplementary Fig. S4). Similar phenotypic changes of MDSC have been found in tumor-bearing mice treated with a TLR9 ligand or recombinant IFN-α and were associated with a reduced suppressive function (32).

High serum levels of TGF-β correlate with poor prognosis and resistance to therapy in patients with pancreatic cancer (33). We previously reported elevated TGF-β serum levels in mice with Panc02 tumors (34). To document the influence of Panc02 tumors on TGF-β serum levels, we analyzed serum samples on days 0, 14, and 25 after tumor induction. TGF-β serum levels were increased in tumor-bearing animals and correlated with tumor burden (Fig. 5D). *In vivo* administration of both, OH-TGF-β or ppp-TGF-β, significantly reduced serum TGF-β levels in mice with early- and late-stage pancreatic tumors (Fig. 5E). Together, these results confirm the *in vivo* activity of ppp-TGF-β in regards to systemic TGF-β silencing and RIG-I activation in mice with orthotopic pancreatic cancer.

Systemic treatment of mice with ppp-TGF-β induces a T_H1 cytokine profile, $CD8^+$ T cell activation, and apoptosis in tumor tissue

Next, we addressed the question whether systemic treatment with ppp-TGF-β results in TGF-β silencing and RIG-I activation in pancreatic tumor tissue *in vivo*. Mice with orthotopic Panc02 tumors were treated with RNAs on days 12 and 14 after tumor induction, and tumors were removed 12 hours later for *ex vivo* analysis. Both, OH-TGF-β and ppp-TGF-β significantly reduced TGF-β on mRNA and protein levels (Fig. 6A). Moreover, bifunctional ppp-TGF-β induced upregulation of CXCL10 and IFN-β expression in tumor tissue (Fig. 6B). To further characterize the cytokine milieu in tumors, we measured levels of interleukin (IL)-4, IL-5, and IFN-γ expression by qRT-PCR. Interestingly, OH-TGF-β and ppp-TGF-β significantly reduced the levels of IL-4 and IL-5. In addition, ppp-TGF-β enhanced IFN-γ expression, indicative of a shift from a T helper T_H2 toward a T_H1 immunoresponse (Fig. 6C). No difference in FoxP3 expression, a marker expressed by regulatory T cells, was observed between treatment groups (Fig. 6C).

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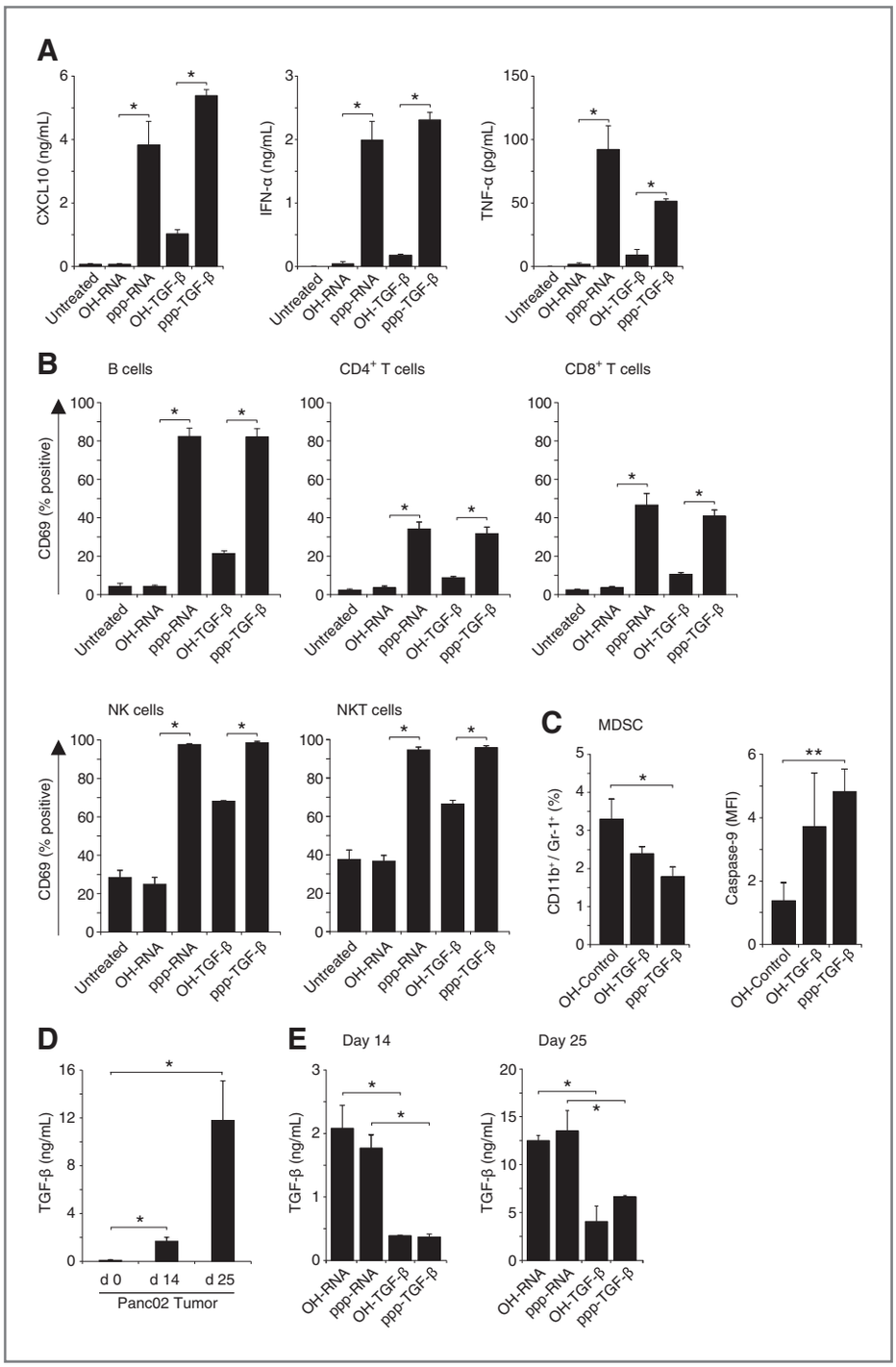

Figure 5. Bifunctional ppp-siRNA targeting TGF-β_1 induces systemic immune activation and gene silencing *in vivo*. On day 10 after Panc02 tumor induction, mice were injected with 50 μg of the indicated RNAs complexed with *in vivo* jetPEI via the tail vein. A, CXCL10, IFN-α, and TNF-α serum levels were analyzed by ELISA after 6 hours. B, CD69 expression by splenic lymphocyte populations was determined by flow cytometry after 12 hours. C, percentage and caspase-9 activation of $CD11b^+$ $Gr\text{-}1^+$ MDSC were measured by flow cytometry after 12 hours. D, TGF-β serum levels in mice during Panc02 tumor progression on days 0, 14, and 25 after tumor induction. E, influence of treatment with RNAs with and without TGF-β silencing activity on TGF-β serum levels at days 14 and 25. Mice were treated twice weekly and serum was taken 48 hours after the second and fifth RNA injections. Pooled data of 3 to 7 mice per group are shown as mean + SEM. *, $P < 0.05$.

H&E and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining of tumor sections revealed profound tumor cell apoptosis in mice treated with ppp-TGF-β (Fig. 7A and B), which correlated with increased caspase-9 activity in tumor lysates (Fig. 7C). Immunohistology and FACS analysis revealed increased numbers of tumor-infiltrating $CD8^+$ T cells and upregulation of the activation marker CD69 (Fig. 7D). Together, these data show potent antitumor activity of ppp-TGF-β treatment *in vivo*.

Therapy with ppp-TGF-β controls pancreatic tumor growth in a $CD8^+$ T cell–dependent manner

Finally, we assessed the *in vivo* efficacy of ppp-TGF-β treatment in regards to survival in mice with orthotopic

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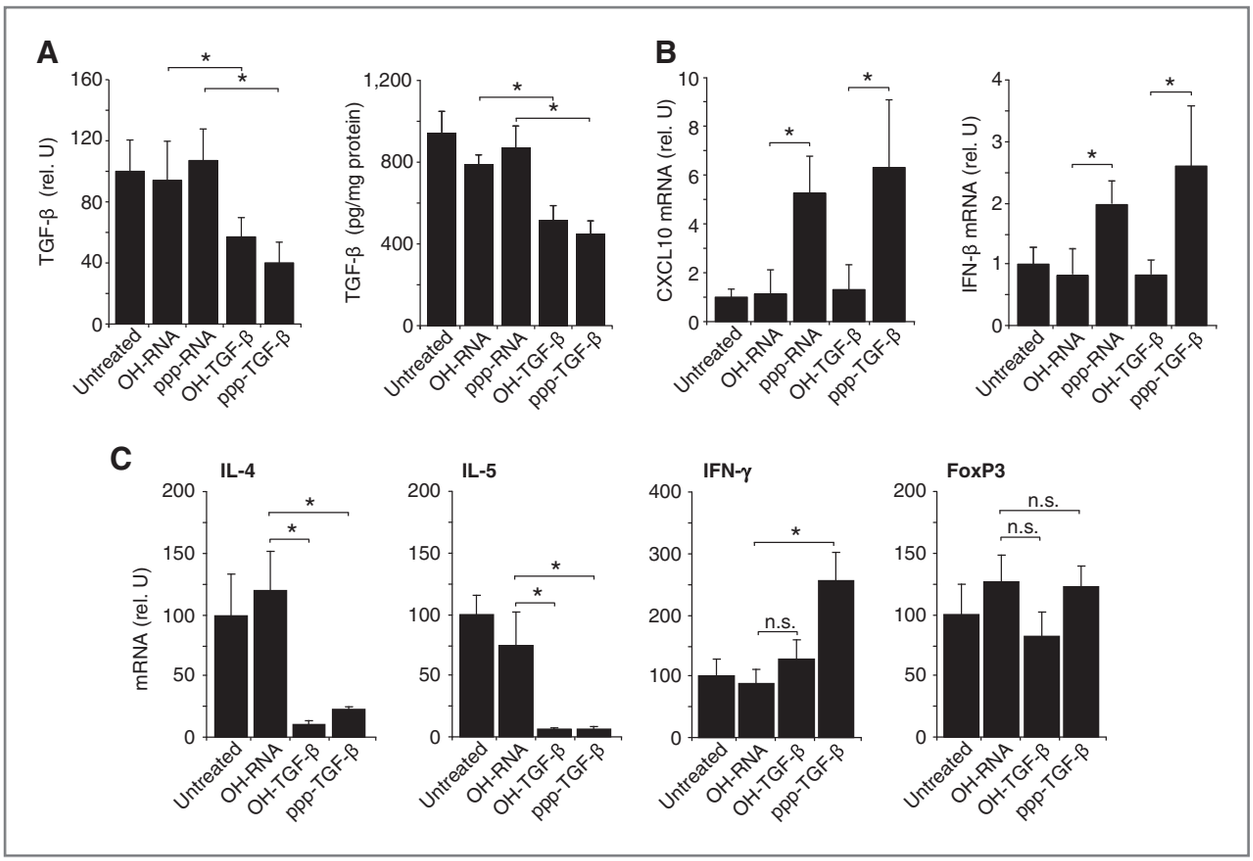

Figure 6. Treatment with ppp-TGF-β induces TGF-β silencing, type I IFN induction, and a T_H1 cytokine profile in tumor tissue. Mice with Panc02 tumors were treated twice with 50 μg RNA as indicated. A, expression levels of TGF-β in tumor tissue were assessed by qRT-PCR and ELISA. B and C, expression levels of CXCL10, IFN-β, IL-4, IL-5, IFN-γ, and FoxP3 in tumor tissue were measured by qRT-PCR. Data represent mean + SEM of pooled data from 5 mice/group. *, $P < 0.05$.

Panc02 tumors. We treated mice with RNAs twice weekly for 3 weeks, starting on day 10 after tumor induction. All mice without treatment or treated with nonsilencing OH-RNA had to be sacrificed because of progressive tumor growth within 40 days after tumor induction (median survival 31 and 29 days, respectively). Treatment with OH-TGF-β or ppp-RNA without silencing activity significantly prolonged survival (median survival 43 and 39 days, respectively). Most efficient tumor control was achieved by bifunctional ppp-TGF-β with a median survival time of 49 days (OH-RNA vs. ppp-TGF-β < 0.0001; OH-TGF-β vs. ppp-TGF-β < 0.05)). Complete tumor regressions, confirmed at autopsy on day 100 after tumor induction, were 0%, 6%, and 33% for OH-RNA, ppp-RNA, OH-TGF-β, and ppp-TGF-β, respectively (Fig. 7E).

Increased infiltrations of tumors with activated $CD8^+$ T cells led us to hypothesize that long-term tumor regression induced by ppp-TGF-β treatment may reflect the induction of an adaptive immunoresponse against Panc02 tumor cells. We therefore analyzed the role of $CD8^+$ T cells as well as NK cells in the treatment response by injecting either α-CD8 or α-NK1.1 depleting mAb before RNA therapy. Depletion of $CD8^+$ T cells substantially reduced the therapeutic efficacy of ppp-TGF-β, whereas depletion of NK cells had no major effect on tumor control in this model (Fig. 7F). Thus, $CD8^+$ T cells seem to be the main effector cells for ppp-TGF-β–induced tumor control.

We next evaluated toxicity of RNA treatment by monitoring blood cell counts, creatinine, urea, lactate dehydrogenase, and alanine aminotransferase serum levels. We observed a transient leukopenia in mice treated with either OH-TGF-β or ppp-TGF-β, which was completely reversible within 48 hours. No obvious signs of therapy-associated distress or organ toxicity were detected by serum chemistry or histopathology (Supplementary Fig. S5 anddata not shown).

Discussion

Sequence-specific degradation of viral RNA by RNAi and innate antiviral responses upon detection of viral nucleic acids by pattern recognition receptors, such as RIG-I, are 2 major antiviral defense mechanisms preceding the development of an adaptive immunoresponse. The requirements for the elimination of virus-infected cells and tumor cells share many features. Here, we use both antiviral principles for the therapy

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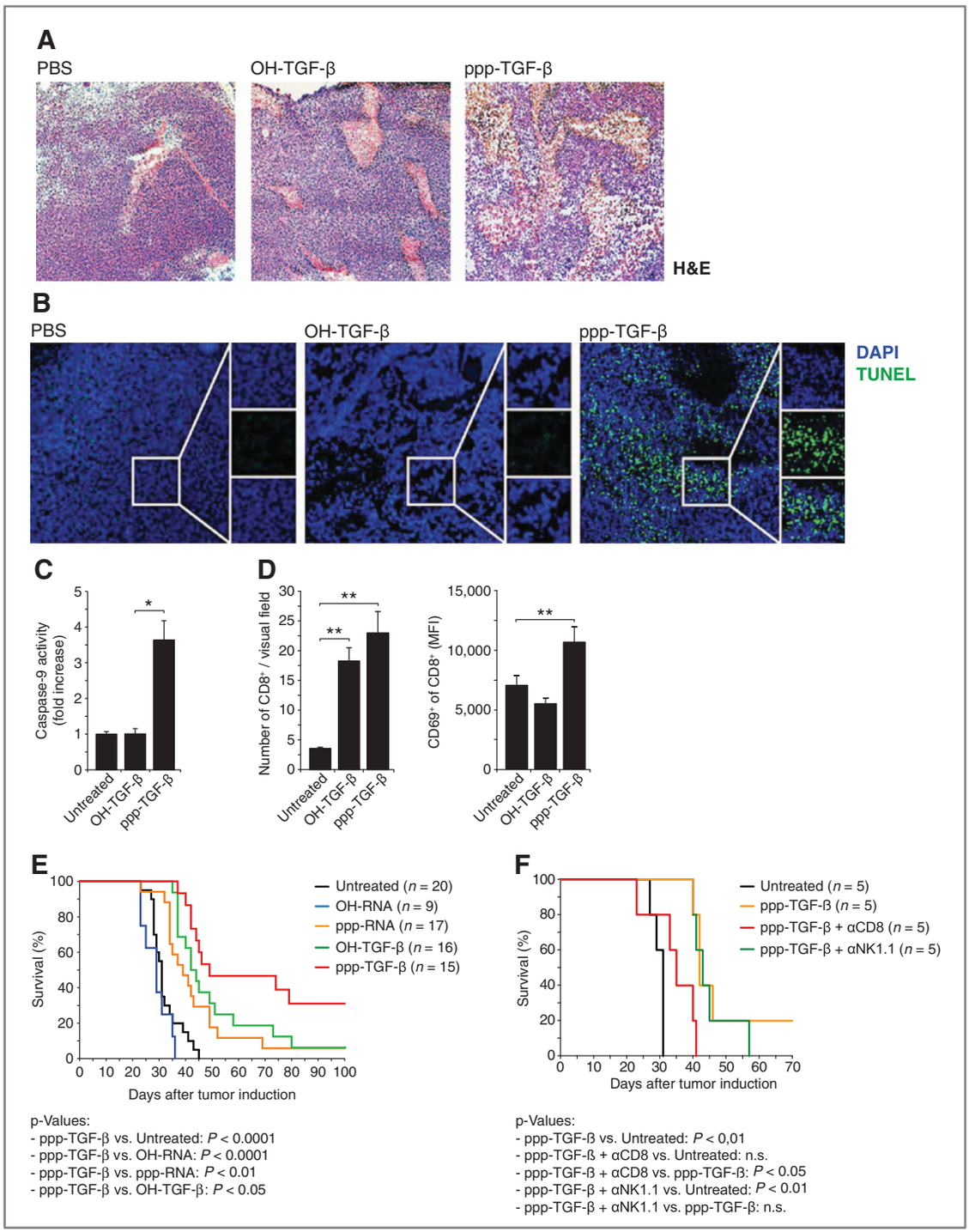

Figure 7. Treatment with ppp-TGF-β induces tumor cell apoptosis *in vivo* and prolongs survival of mice with Panc02 tumors in a $CD8^+$ T cell–dependent manner. A–C, mice with Panc02 tumors were treated with 50 μg of the indicated RNA on days 12 and 14 after tumor induction. Tumors were removed on day 15 for *ex vivo* analysis using H&E staining (A) and TUNEL staining (B) for detecting apoptotic tumor cells (green) in cryosections of tumor tissue. Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI; blue). Representative pictures of 5 tumors per group. C, caspase-9 activity in tumor tissue was assessed by colorimetric analysis. D, number and CD69 expression of tumor-infiltrating $CD8^+$ T cells as assessed by histology and flow cytometry, respectively. Data represent mean + SEM from 5 mice. *, $P < 0.05$. E, survival of mice with orthotopic Panc02 tumors treated with RNA twice weekly for 3 weeks was monitored. Treatment started on day 10 after tumor induction. Experiments were terminated after 100 days (all surviving mice were tumor-free). Pooled data with statistical analysis from 4 independent experiments with 9 to 20 mice per group are depicted. F, survival of mice with orthotopic Panc02 tumors treated with RNA in the absence or presence of depleting mAb against CD8 or NK1.1 was monitored. Data from 5 mice per group with statistical analysis are depicted.

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of pancreatic cancer. We show that functional RIG-I is expressed in human pancreatic cancer cells. Furthermore, a designed RNA molecule to conduct both RIG-I activation and silencing of the immunosuppressive cytokine TGF-β induced tumor regression in a $CD8^+$ T cell–dependent manner in an aggressive mouse model of pancreatic cancer. In this respect, RNA molecules that contained either the RIG-I ligand motif or the silencing capability alone were less effective supporting the concept that bifunctional siRNA is superior for effective tumor therapy.

A major hurdle in cancer immunotherapy is the profound-immunosuppression, both systemically and locally within the tumor microenvironment. RIG-I signaling leads to type I IFN responses with IFN-dependent gene products triggering innate and adaptive immunoresponses (18). These effects include systemic activation of NK cells and T cells, as well as activation of dendritic cells, which are critical for the induction and regulation of adaptive immunoresponses and play a key role in cancer immune surveillance (35). It is important to note that upon RIG-I activation, type I IFN not only derives from immune cells but also from tumor tissue, as shown for human and murine pancreatic cancer cells in our work. Secretion of CXCL10 can attract lymphocytes to tumor tissue, and locally produced IFN-β can activate T_H1 responses and tumor-infiltrating CTL thus enhancing their killing function. In this respect, upregulation of MHC-I expression by the tumor cells upon RIG-I activation may promote CTL-mediated tumor recognition and killing. In fact, we observed that treatment with ppp-TGF-β resulted in a T_H1 cytokine profile in tumor tissue and a more vigorous tumor infiltration with activated $CD8^+$ T cells. In addition, we found that tumor regression in response to ppp-TGF-β treatment was mediated by $CD8^+$ T cells. This leads to the question of how ppp-TGF-β restored $CD8^+$ T cell responses in tumors.

Soluble factors, such as TGF-β (5, 6), and immune cell populations, such as MDSCs and regulatory T cells, have been shown to play immunosuppressive roles in pancreatic cancer (30, 36). In our study, treatment with ppp-TGF-β effectively reduced TGF-β levels in both serum and tumors. The frequency of regulatory T cells was not influenced, however, we have previously reported that TGF-β silencing results in a marked downregulation of CD103 expression on regulatory T cells (37). As CD103 identifies a particularly suppressive subtype of regulatory T cells, treatment with ppp-TGF-β may counteract regulatory T cell–mediated CTL suppression. In addition, we observed that ppp-TGF-β significantly reduced the numbers of $CD11b^+$ $Gr\text{-}1^+$ MDSCs. Because MDSCs are frequently found in pancreatic cancer tissue and potently suppress $CD8^+$ T cells, this finding is particularly interesting (36). Moreover, MDSC underwent phenotypic changes, such as upregulation of CD11c, CD80, and Sca-1 expression. Interestingly, similar changes in MDSC have been reported in tumor-bearing mice treated with recombinant IFN-α and were found to correlate with a reduced T cell–suppressive function (32). Thus, TGF-β silencing and type I IFN induction induced by ppp-TGF-β seem to have additive effects on breaking the immunosuppressive milieu created by pancreatic cancer cells and are capable of tipping the balance toward effective antitumor CTL responses.

A central aspect of ppp-RNA treatment is the induction of tumor cell apoptosis. Pancreatic cancer cells frequently acquire loss-of-function mutations of the gatekeeper protein p53, which reduces their sensitivity toward proapoptotic signals (38). An elegant strategy to circumvent this limitation is the exploitation of p53-independent apoptosis induction. We found that pancreatic carcinoma cell lines, including those with p53 mutations (PANC-1, BxPC-3, and MIA-PaCa-2), were sensitive to ppp-RNA–mediated apoptosis. In line with findings in melanoma (14), we found that ppp-RNA triggers apoptosis via the mitochondrial pathway in pancreatic cancer cells involving upregulation of the BH3-only proteins Noxa and Puma with subsequent caspase-9 activation. Moreover, systemic treatment with ppp-TGF-β induced profound tumor cell apoptosis *in vivo*, whereas normal pancreas (as well as other organs, such as liver, kidney, and lung) showed no signs of histopathology. These findings confirm previous reports that tumor cells are highly susceptible to ppp-RNA–induced apoptosis (14). The predilection for tumor cells as compared with healthy tissue is critical for avoiding toxicity and provides a therapeutic window for ppp-RNA treatment.

In conclusion, we identified RIG-I as a novel target for immunotherapy of pancreatic cancer. Combining RIG-I activation with TGF-β silencing via bifunctional ppp-siRNA breaks tumor-mediated immunosuppressive mechanisms and confers potent antitumor efficacy. Whether this strategy can be further improved, for example, by combination with cytotoxic agents or immunization, is the focus of ongoing studies. Further improvement can be expected by designing new delivery systems for selective tumor targeting and by assessment in genetically engineered mouse models of pancreatic cancer, which allow studying effects on the tumor stromal compartment and metastatic spreading (39, 40).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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6. Veröffentlichungen

6.1 Originalarbeiten

1. David Anz*, Wolfgang Müller*, Michaela Golic, Wolfgang G. Kunz, Moritz Rapp, Viktor H. Koelzer, **Jonathan Ellermeier**, Joachim W. Ellwart, Max Schnurr, Carole Bourquin, Stefan Endres.
   CD103 is a hallmark of tumor-infiltrating regulatory T cells.
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   **contributed equally*

6.2 *Abstracts* und Vorträge

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   Treatment of pancreatic carcinoma with a 5'-Triphosphate siRNA targeting the immunosuppressive molecule TGFβ1.
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