Oznaka poročila: ARRS-RPROJ-ZP-2012/18

ZAKLJUČNO POROČILO O REZULTATIH RAZISKOVALNEGA PROJEKTA

A. PODATKI O RAZISKOVALNEM PROJEKTU

1.Osnovni podatki o raziskovalnem projektu

Šifra projekta	Z7-2059
Naslov projekta	Aktivacija imunskega odziva z naravnimi in sintetičnimi nanodelci
Vodja projekta	23563 Iva Hafner Bratkovič
Tip projekta	Zt Podoktorski projekt - temeljni
Obseg raziskovalnih ur	3400
Cenovni razred	B
Trajanje projekta	05.2009 - 04.2011
Nosilna raziskovalna organizacija	104 Kemijski inštitut
Raziskovalne organizacije - soizvajalke	
Raziskovalno področje po šifrantu ARRS	7 INTERDISCIPLINARNE RAZISKAVE
Družbeno-ekonomski cilj	07. Zdravje

2.Raziskovalno področje po šifrantu FOS[1]

Šifra	3.01
- Veda	3 Medicinske vede
- Področje	3.01 Temeljna medicina

B. REZULTATI IN DOSEŽKI RAZISKOVALNEGA PROJEKTA

3.Povzetek projekta[2]

SLO

Odlaganje amiloidnih oblik drugače topnih proteinov je karakteristično za amiloidne bolezni kot so Alzheimerjeva in Parkinsonova bolezen ter prionske bolezni. Te bolezni spremlja tudi vnetje, trenutno pa ni jasno, kaj je dejanski vzrok nevrodegeneracije. Pred kratkim so pokazali, da agregati peptida Aβ aktivirajo znotrajcelični receptor NLRP3

(NALP3), kar povzroči aktivacijo kaspaze-1 in sproščanje IL-1β iz celic mikroglije. V tem projektu smo raziskali mehanizem sproščanja nevrotoksičnih oziroma provnetnih zvrsti iz celic mikroglije in makrofagov, ki jih povzročijo naravni in sintetični nanodelci. Raziskali smo vlogo inflamasomov v prionskih boleznih in sinukleinopatijah. Pri različnih prionskih boleznih so opazili povečano izražanje IL-1β. Pokazali so tudi, da miši z izbition genom za IL-1R za prionsko boleznijo zbolijo kasneje kot miši divjega tipa. Mi pa smo pokazali, da dodatek fibril iz prionskega proteina k celicam mikroglije ali makrofagom povzroči sproščanje zrele oblike IL-1β, kar je odvisno od NLRP3-inflamasoma. Ta proces je odvisen tudi od znižanja znotrajcelične koncentracije K^+, potrebno pa je tudi, da pride do fagocitoze fibril, ki posledično vodi do destabilizacije lizosomov. Ugotovili smo, da so fibrile najmočnejši aktivator inflamasoma med definiranimi različnimi oblikami prionskega proteina, sledijo agregati, medtem ko nativna monomerna oblika in oligomeri ne aktivirajo NLRP3-inflamasoma. Naši rezultati skupaj s predhodnimi študijami na miših z izbitim genom za IL-1R predlagajo IL-1 signalno pot kot perspektivno tarčo za terapijo prionskih bolezni.

ANG

A characteristic of amyloid diseases, such as Alzheimer's, Parkinson's and prion diseases is the accumulation of amyloid form of otherwise soluble proteins. This type of diseases is also accompanied by inflammation and it is currently unclear, what is the underlying cause of neuronal degeneration. Recently it has been shown that Aβ peptide aggregates activate the intracellular NLRP3 (NALP3) receptor, which leads to the activation of caspase-1 and release of IL-1β from microglial cells. In this project we investigated the mechanism of microglia and macrophage release of neurotoxic and proinflammatory species by natural and synthetic nanoparticles. We explored the role of inflammasomes in prion disease and synucleinopathies. Elevated expression of the proinflammatory cytokine IL-1β has been observed in brains affected by several prion diseases and IL-1R-deficiency significantly prolonged the onset of the neurodegeneration in mice. We showed that microglia and macrophages release IL-1β upon stimulation by PrP fibrils, which depends on the NLRP3 inflammasome. Activation of NLRP3 inflammasome by PrP fibrils requires depletion of intracellular K^+ and requires phagocytosis of PrP fibrils and consecutive lysosome destabilization. Among the well-defined molecular forms of PrP the strongest NLRP3 activation was observed by fibrils, followed by aggregates, while neither native monomeric nor oligomeric PrP were able to activate the NLRP3 inflammasome. Our results together with previous studies on IL-1R-deficient mice suggest the IL-1 signaling pathway as the perspective target for the therapy of prion disease.

4.Poročilo o realizaciji predloženega programa dela na raziskovalnem projektu[3]

Cilja projekta sta bila 1) raziskati ali naravni in sintetični nanodelci povzročajo vnetje z aktivacijo inflamasomov, predvsem NLRP3-inflamasoma, ter 2) kakšen je mehanizem te aktivacije.
Najprej smo se osredotočili na vlogo NLRP3-inflamasoma pri nevrodegenerativnih boleznih, kot so prionske bolezni in Parkinsonova bolezen. Pri teh boleznih pride do odlaganja prionskega proteina oziroma alfa-sinukleina, zato smo v bakterijah proizvedli prionski protein in alfa-sinuklein. Pri prionskih boleznih se nativna, alfa-oblika, pretvori v patološko obliko. S pomočjo uvedenih disulfidnih mostičkov smo raziskali tudi strukturno spremembo prionskega proteina in pokazali, da pride do pretvorbe le v primeru, ko je možna ločitev poddomen B1-H1-B2 in H2-H3 in nastane dimer z izmenjavo domen (Hafner Bratkovič in sod., J Biol Chem, 2011 in Hafner Bratkovič in Jerala, Prion, 2011, podrobnejši opis znanstveni dosežek 1). Rigidna zanka je zanka, ki povezuje B2 in H2 in je odgovorna za medvrstno bariero. Naši rezultati nakazujejo, da je Q/N zaporedje v rigidni zanki pomembno za pretvorbo sesalskega prionskega proteina in morda deluje podobno kot pri kvasnih prionih in poliglutaminskih boleznih, kot je Huntingtonova bolezen (Avbelj, Hafner Bratkovič in Jerala, BBRC, 2011, podrobnejši opis znanstveni dosežek 4) . Med prionske bolezni uvrščamo tudi dedne oblike, katerih vzrok je v mutacijah v genu za prionski protein. Zanimivo je, da je pribiližno tretjina patoloških mutacij v globularnem predelu taka, da pride do zamenjave z bolj hidrofobnim aminokislinskim ostankom. Zanimalo nas je, če lahko

morda z uvedbo hidrofobnih aminokislinskih ostankov ojačamo hidrofobne interakcije znotraj poddomen, kar pa ne bi smelo negativno vplivati na ločitev poddomen in pretvorbo. Pokazali smo, da mutanta T187I tvori več amiloida *in vitro* in v okužnih celičnih linijah več oblike prionskega proteina, odporne proti razgradnji s proteinazo K. Pod mikroskopom na atomsko silo smo pokazali, da pri tej mutanti med fibrilizacijo nastajajo tanjše fibrile oziroma filamenti, ki lahko predstavljajo nova jedra za fibrilizacijo (Hafner Bratkovič in sod., Plos One, 2011; podrobnejši opis znanstveni dosežek 2).

Prionski protein obstaja v različnih oblikah. Pri zdravih ljudeh je prisotna nativna monomerna alfa-oblika, ki pa se v bolezenskih stanjih pretvori v obliko s povečanim deležem beta sekundarne strukture, takšni so npr. beta-oligomeri in amiloidne fibrile. Pokazali smo, da fibrile v prisotnosti celic mikroglije povzročijo nevronsko smrt, saj celice mikroglije spodbudijo k izločanju nevrotoksičnih zvrsti. Pripravili smo različne strukturne oblike prionskega proteina: nativno (alfa-obliko), beta-oligomer, amiloidne fibrile in amorfne agregate, saj nas je zanimalo, če lahko aktivirajo makrofage. Ugotovili smo, da amiloidne fibrile prionskega proteina povzročijo koncentracijsko odvisno izločanje IL-1b (IL-1beta) iz celic mikroglije in makrofagov. V manjši meri povzročijo izločanje IL-1b tudi agregati. Nativna oblika in oligomeri pa ne povzročijo izločanja zrele oblike IL-1b. Nato smo pokazali, da je s prionskimi fibrilami inducirano izločanje IL-1b odvisno od kaspaze-1 in tudi od NLRP3-inflamasoma. Pri aktivaciji NLRP3-inflamasoma pride do sestavljanja inflamasomskega kompleksa iz receptorja NLRP3, adapterske molekule ASC in pro-kaspaze-1. Pri tem kaspaza-1 aktivira sama sebe in cepi pro-IL-1b. Zrela oblika IL-1b se nato izloča iz celice in deluje provnetno preko receptorja IL-1R. Za določitev mehanizma izločanja IL-1b smo uporabili makrofagne in mikroglijske celice z izbitim genom za ASC, NLRP3, kaspazo-1 in IL-1R. Celice z izbitimi geni za komponente NLRP3-inflamasoma se na fibrile prionskega proteina niso odzvale, medtem ko so se celice divjega tipa in celice z izbitim genom za IL-1R odzvale z izločanjem IL-1b. S tem smo pokazali, da fibrile prionskega proteina aktivirajo NLRP3-inflamasom. Podobno smo pokazali tudi za fibrile alfa-sinukleina ter za sintetične nanodelce. Raziskali smo tudi mehanizem aktivacije NLRP3-inflamasoma s proteinskimi in sintetičnimi nanodelci. Do sedaj predpostavljeni mehanizmi aktivacije inflamasoma so znižanje znotrajcelične koncentracije K^+, tvorba reaktivnih kisikovih spojin in destabilizacija lizosomov, ki sledijo fagocitozi kristaliničnih aktivatorjev. Zanimivo je, da z inhibicijo različnih predpostavljenih poti aktivacije dosežemo zmanjšan odziv NLRP3-inflamasoma na fibrile prionskega proteina. Tako npr. inflamasomski odziv na fibrile inhibirajo gliburid (inhibitor kalijevih kanalov), citohalasin D (inhibitor fagocitoze) ter inhibitor NADPH oksidaze in antioksidanti. Za inhibitorje oksidativnega stresa so Bauernfeind in sod. lani pokazali, da ne inhibirajo sestavljanja inflamasomskega kompleksa, ampak inhibirajo prvo stopnjo, to je produkcijo pro-IL-1b in receptorja NLRP3, tako smo to pot prenehali preučevati (Bauernfeind in sod., J Immunology, 2011). Pokazali pa smo tudi, da je za aktivacijo inflamasoma pomembna internalizacija (fagocitoza) fibril, ki privede do zatekanja lizosomov in iztekanja vsebine lizosomov v citosol. Na podlagi naših rezultatov sklepamo, da so fibrile dober aktivator inflamasoma, ker se dobro internalizirajo, so pa slabo razgradljive in privedejo do destabilizacije lizosomov, veliki agregati se slabo internalizirajo, oligomeri in monomer pa ne povzročijo destabilizacije lizosomov.

Nekaj študij je pokazalo, da npr. aktivatorji TLRjev podaljšajo inkubacijo pri mišjih modelih prionskih bolezni, kar pomeni, da nespecifična inhibicija imunskega sistema ne bi bila ustrezna. Naši rezultati in rezultati skupin, ki so raziskovale na mišjih modelih pa nakazujejo, da bi lahko specifična inhibicija signalne poti IL-1 ugodno vplivala na potek prionskih bolezni.

V maju 2011 je bilo to delo (Aktivacija NLRP3-inflamasoma s fibrilami prionskega proteina) predstavljeno v obliki plakata na mednarodni konferenci o naravni imunosti Toll 2011 (Gardsko jezero, Italija), oktobra 2011 kot kratka predstavitev na konferenci z mednarodno udeležbo v organizaciji SBD in SMD, rokopis (priložen v priponki) pa je v

recenzijskem postopku. Raziskave aktivacije inflamasoma z alfa-sinukleinom ter s sintetičnimi nanodelci zaključujemo. V zvezi s tem projektom sodelujemo z vodilnimi znanstveniki na področju naravne imunosti (K. Fitzgerald, D. Golenbock; ZDA) ter prionskih bolezni in sinukleinopatij (I. Vorberg, Nemčija).

5.Ocena stopnje realizacije programa dela na raziskovalnem in zastavljenih raziskovalnih ciljev[4]

Kot je razvidno iz tabele, so bile naloge izvedene in zastavljena cilja dosežena.

CILJI/ NALOGE	REALIZIRANO
Cilj 1: Testiranje citotoksičnosti in aktivacije naravne imunosti z nanodelci	**DA, CILJ DOSEŽEN**
Izražanje in izolacija rekombinantnih PrP	DA
Priprava različnih strukturnih oblik PrP	DA
Priprava amiloidnih fibril iz drugih proteinov	DA
Karakterizacija različnih oblik proteinov	DA
Karakterizacija sintetičnih nanodelcev	DA
Spremljanje viabilnosti celic s testom WST-1	DA (uporabili smo podoben test XTT)
Določitev aktivacije kaspaze-1 in sproščanja IL-1β	DA
Določitev NO in TNF, ki jih ob stiku nanodelcev izločajo mikroglijske celice/ makrofagi	DA
Nevrotoksičnost	DA
Aktivacija Nalp3	DA
Cilj 2: Določitev mehanizma aktivacije inflamasoma z nanodelci	**DA, CILJ DOSEŽEN**
Pomen fagocitoze za citotoksičnost	DA
Pomen destabilizacije lizosomov pri vnetju	DA
Katere proteaze so vpletene v aktivacijo Nalp3?	DA
Vloga oksidativnega stresa pri aktivaciji inflamasoma	DA
Vloga znižanja znotrajcelične koncentracije K^+ pri aktivaciji inflamasoma	DA

6.Utemeljitev morebitnih sprememb programa raziskovalnega projekta oziroma sprememb, povečanja ali zmanjšanja sestave projektne skupine[5]

Ni bilo večjih sprememb programa.

7.Najpomembnejši znanstveni rezultati projektne skupine[6]

	Znanstveni dosežek			
1.	COBISS ID		4602394	Vir: COBISS.SI
	Naslov	*SLO*	Pri patološki pretvorbi prionskega proteina pride z ločitvjo poddomen znotraj strukturirane domene do tvorbe dimera z izmenjavo domen	
		ANG	Globular domain of the prion protein needs to be unlocked by domain	

			swapping to support prion protein conversion
	Opis	SLO	Prionske bolezni so smrtne nevrodegenerativne bolezni ljudi in živali. Normalna oblika prionskega proteina se pretvori v patološko agregirano obliko PrPSc, katere strukture ni mogoče raziskovati s strukturnimi tehnikami visoke resolucije. Odločili smo se, da bomo različne predele prionskega proteina omejili z disulfidnimi mostički in tako opredelili njihovo vlogo pri strukturni pretvorbi. Pri večini mutant smo opazili povečano termično stabilnost, pa so se kljub temu učinkovito pretvorile v fibrilarno obliko. Samo disulfidi, ki so povezali poddomeni B1-H1-B2 in H2-H3 so onemogočili strukturno pretvorbo in vitro in v celičnih modelih prionskih bolezni. Če smo te disulfidne mostičke z redukcijo porušili, so se mutante učinkovito pretvorile, kar nakazuje, da je ločitev poddomen nujen korak pri strukturni pretvorbi prionskega proteina. Pripravili smo tudi mutante, pri katerih so bili enojni cisteini uvedeni tako, da bi tvorili z disulfidom povezane dimere le v primeru dimera z izmenjavo domen, kar smo tudi eksperimentalno dokazali. V nasprotju s predhodnimi modeli PrPSc, ki predvidevajo, da pride do pretvorbe obstoječih elementov sekundarne strukture, smo pokazali, da je velika večina le-teh ohranjenih. Predhodne študije so že nakazale, da je dimerizacija stopnja, ki določa hitrost fibrilizacije PrP, mi pa smo pokazali, da sta ločitev in izmenjava poddomen znotraj strukturirane domene nujni za pretvorbo in predlagamo mehanizem, pri katerem pred amiloidogenezo najprej nastane dimer z izmenjavo domen, ki bi lahko bil pomembna stopnja za inhibicijo strukturne pretvorbe.
		ANG	Prion diseases are fatal transmissible neurodegenerative diseases affecting many mammalian species. The normal prion protein (PrP) converts into a pathological aggregated form, PrPSc, which is enriched in the beta-sheet structure. While the high resolution structure of the normal PrP was determined, the structure of the converted form of PrP remains inaccessible to high resolution techniques. In order to map the PrP conversion process we introduced disulfide bridges into different positions within the globular domain of PrP, tethering selected secondary structure elements. The majority of tethered PrP mutants exhibited increased thermodynamic stability, nevertheless they converted efficiently. Only the disulfides which tether subdomain B1-H1-B2 to subdomain H2-H3 prevented PrP conversion in vitro and in prion infected cell cultures. Reduction of disulfides recovered the ability of these mutants to convert, demonstrating that the separation of subdomains is an essential step in conversion. Formation of disulfide-linked proteinase K-resistant dimers in fibrils composed of a pair of single cysteine mutants supports the model based on domain-swapped dimers as the building blocks of prion fibrils. In contrast to previously proposed structural models of PrPSc suggesting conversion of large secondary structure segments, we provide evidence for the conservation of secondary structure elements of the globular domain upon PrP conversion. Previous studies already showed that dimerization is the rate-limiting step in PrP conversion. We show that separation and swapping of subdomains of the globular domain is necessary for conversion. Therefore, we propose that domain-swapped dimer of PrP precedes amyloid formation and represents a potential target for therapeutic intervention.
	Objavljeno v		American Society of Biological Chemists.; The Journal of biological chemistry; 2011; Vol. 286, no. 14; str. 12149-12156; Impact Factor: 5.328;Srednja vrednost revije / Medium Category Impact Factor: 3.787; A': 1; WoS: CQ; Avtorji / Authors: Hafner Bratkovič Iva, Bester Romina, Pristovšek Primož, Gaedtke Lars, Veranič Peter, Gašperšič Jernej, Manček Keber Mateja, Avbelj Matevž, Polymenidou Magdalini, Julius Christian, Aguzzi Adriano, Vorberg Ina, Jerala Roman
	Tipologija		1.01 Izvirni znanstveni članek
2.	COBISS ID		4764442 Vir: COBISS.SI

	Naslov	*SLO*	Vpliv hidrofobnih mutacij v poddomeni H2-H3 na stabilnost in pretvorbo prionskega proteina in vitro in in vivo
		ANG	Effect of hydrophobic mutations in the H2-H3 subdomain of prion protein on stability and conversion In Vitro and In Vivo
	Opis	*SLO*	Prionske bolezni so usodne nevrodegenerativne bolezni, ki so lahko pridobljene, sporadične ali dedne. Pri približno eni tretjini patoloških mutacij se zamenja aminokislinski ostanek za bolj hidrofobnega, zato nas je zanimalo, kako ojačanje hidrofobnih interakcij v pod-domeni H2-H3 vpliva na pretvorbo prionskega proteina. V mišji prionski protein smo uvedli tri točkovne hidrofobne mutacije. Človeška patološka mutacija V209I ni vplivala na stabilnost in fibrilizacijo PrP, medtem ko sta dizajnirani mutaciji V175I and T187I povečali termično stabilnost PrP. Mutanta V175I je fibrilizirala hitreje kot protein divjega tipa. T187I se je pretvarjala počasneje, vendar pa je bila intenziteta fluorescence za amiloide specifičnega barvila tioflavina T signifikantno višja. Z virusno transdukcijo smo pripravili tudi celične linije, ki so izražale te mutante in jih izpostavili mišjim prionom seva 22L. Celice, ki so izražale V209I so slabo podpirale propagacijo prionov, medtem ko so V175I, T187I in protein divjega tipa učinkovito pretvarjali v obliko, odporno proti razgradnji s proteinazo K (PrPRes). Podobno kot pri fibrilizaciji in vitro, smo tudi v celičnih kulturah pri mutanti T187I opazili povečano tvorbo PrPRes v primerjavi s proteinom divjega tipa. Z analizo pod mikroskopom na atomsko silo smo ugotovili, da pri pretvorbi T187I nastajajo tanke fibrile, ki jih pri drugih proteinih nismo opazili. Možno je, da I187 v primerjavi z ostalimi mutacijami onemogoča lateralno pakiranje filamentov in tako povzroča bolj učinkovito pretvorbo. Celične linije z za okužbo bolj dovzetnimi mutantami lahko služijo za razvoj bolj občutljivih prionskih celičnih testov.
		ANG	Prion diseases are fatal neurodegenerative diseases, which can be acquired, sporadic or genetic, the latter being linked to mutations in the gene encoding prion protein. The goal of the present study was to investigate the effect of increasing the hydrophobic interactions within the H2-H3 subdomain on PrP conversion. Three hydrophobic mutations were introduced into PrP. The mutation V209I associated with human prion disease did not alter protein stability or in vitro fibrillization propensity of PrP. The designed mutations V175I and T187I on the other hand increased protein thermal stability. V175I mutant fibrillized faster than wild-type PrP. Conversion delay of T187I was slightly longer, but fluorescence intensity of amyloid specific dye thioflavin T was significantly higher. Surprisingly, cells expressing V209I variant exhibited inefficient proteinase K resistant PrP formation upon infection with 22L strain, which is in contrast to cell lines expressing wild-type, V175I and T187I mPrPs. In agreement with increased ThT fluorescence at the plateau T187I expressing cell lines accumulated an increased amount of the proteinase K-resistant prion protein. We showed that T187I induces formation of thin fibrils, which are absent from other samples. We propose that larger solvent accessibility of I187 in comparison to wild-type and other mutants may interfere with lateral annealing of filaments and may be the underlying reason for increased conversion efficiency. Cell lines expressing more susceptible mutants could be used for development of more sensitive scrapie cell assays.
	Objavljeno v		Public Library of Science; PLoS one; 2011; Vol. 6, iss. 9; str. e24238-1-e24238-9; Impact Factor: 4.411;Srednja vrednost revije / Medium Category Impact Factor: 2.025; A': 1; WoS: CU; Avtorji / Authors: Hafner Bratkovič Iva*, Gaedtke Lars*, Ondračka Andrej*, Veranič Peter, Vorberg Ina, Jerala Roman; * equal contribution
	Tipologija		1.01 Izvirni znanstveni članek
3.	COBISS ID		4377626 Vir: COBISS.SI
			Prionski protein (PrP) s tetracisteinsko oznako omogoča razlikovanje med

	Naslov	*SLO*	nativno in pretvorjeno obliko PrP
		ANG	Tetracysteine-tagged prion protein allows discrimination between the native and converted forms
	Opis	*SLO*	Opisali smo novo metodo za sintezo biarzinskih spojin, za katere je značilno, da se specifično vežejo na tetracisteinsko peptidno oznako. Slednjo smo vnesli v različna področja mišjega prionskega proteina in razvili kvantitativno metodo za spremljanje pretvorbe PrP, ki ne uporablja proteaz, ampak temelji na različni prepoznavi monomerne in patološke oblike z biarzinskim reagentom. Pokazali smo tudi, da lahko s temi reagenti detektiramo s tetracisteinsko oznako označen PrP v celičnih kulturah, kar še razširja možnost uporabe te metode za detekcijo in študij konformacijskih bolezni.
		ANG	We report a new method for the synthesis of biarsenical reagents, which are then used to monitor murine PrP (mPrP) misfolding. We introduced tetracysteine (TC) tags which bind biarsenical compounds into mPrP and devised a quantitative protease-free method for following PrP conversion, based on the ability of the biarsenical reagent to differentiate between the monomeric and fibrilized form of TC-tagged PrP, and showed that TC-tagged mPrP could be detected on transfected cells, thereby expanding the potential use of this method for the detection and study of conformational diseases.
	Objavljeno v		Blackwell; FEBS journal; 2010; Vol. 277, no. 9; str. 2038-2050; Impact Factor: 3.129;Srednja vrednost revije / Medium Category Impact Factor: 3.787; WoS: CQ; Avtorji / Authors: Gašperšič Jernej, Hafner Bratkovič Iva, Stephan Michel, Veranič Peter, Benčina Mojca, Vorberg Ina, Jerala Roman
	Tipologija		1.01 Izvirni znanstveni članek
4.	COBISS ID		4766234 Vir: COBISS.SI
	Naslov	*SLO*	Dodatni glutaminski aminokislinski ostanki v zanki B2-H2 pospešijo pretvorbo prionskega proteina
		ANG	Introduction of glutamines into the B2-H2 loop promotes prion protein conversion
	Opis	*SLO*	Pri prionskih boleznih se celična oblika prionskega proteina (PrPC) konformacijsko pretvori v PrPSc, obliko s povečanim deležem ß-sekundarne strukture. PrPC je sestavljen iz nestrukturiranega N-končnega dela in globularne, C-končne domene, ki vsebuje tri alfa vijačnice (H1, H2, H3) in antiparalelno beta ploskev (B1, B2). Zanka B2-H2, ki ima ključno vlogo pri medvrstni barieri, vsebuje največjo gostoto asparaginskih (N) in glutaminskih (Q) aminokislinskih ostankov v celotnem zaporedju prionskega proteina. S Q/N-obogatene domene so ključne za pretvarjanje prionov iz kvasovk. Raziskali smo vlogo Q/N aminokislinskih ostankov v zanki B2-H2 pri pretvarjanju prionskega proteina. Pripravili smo mutante s povečanim številom zaporednih Q/N v zanki B2-H2. Stabilnost mutant je padala obratno sorazmerno s številom Q/N. Morfologija fibril je bila podobna kot pri fibrilah divjega tipa, faza zakasnitve fibrilizacije pa se je s številom Q/N krajšala. Pokazali smo, da je efekt povečanja Q/N specifičen za zanko B2-H2 in ni posledica večje fleksibilnosti zanke, saj uvedba zaporedja, obogatenega bodisi z glicinskimi in serinskimi bodisi z alaninskimi ostanki namreč kljub zmanjšani stabilnosti podaljša fazo zakasnitve fibrilizacije. Naši rezultati demonstrirajo, da Q/N zaporedje v zanki B2-H2 promovira pretvorbo in morda predstavlja povezavo s Q/N-prioni (kvasnimi prioni).
			In prion diseases cellular prion protein (PrPC) undergoes conformational transition into the ß-sheet-rich form (PrPSc). PrPC consists of the disordered N-terminal part and a C-terminal globular domain containing three alpha helices (H1, H2, H3) and an antiparallel beta sheet (B1, B2).

		ANG	B2-H2 loop, which has a focal role in the species barrier, contains the highest density of asparagine (N) and glutamine (Q) residues in the whole sequence. Q/N-rich domains are essential for the conversion of yeast prions. We investigated the role of Q/N residues in the B2-H2 loop in PrP conversion. We prepared mouse PrP mutants with increasing number of consecutive Q/N residues in the B2-H2 loop. Stability of the mutants decreased with the increasing number of inserted glutamines. In vitro conversion of mutants yielded fibrils of similar morphology as the wild-type PrP. Q/N mutants accelerated fibrillization in comparison to the wild-type PrP, with mutant containing the most glutamines having the shortest lag phase. The effect of Q/N residues was specific for the B2-H2 loop and was not due to simple increase in flexibility as the introduction of Gly-Ser or Ala residues slowed the conversion despite their decreased stability. Our results thus suggest that Q/N residues in the B2-H2 loop of PrP promote protein conversion and may represent a link to conversion of Q/N-rich prions.
	Objavljeno v		Academic Press.; Biochemical and biophysical research communications; 2011; Vol. 413, issue 3; str. 521-526; Impact Factor: 2.595;Srednja vrednost revije / Medium Category Impact Factor: 3.337; WoS: CQ, DA; Avtorji / Authors: Avbelj Matevž*, Hafner Bratkovič Iva*, Jerala Roman *equal contribution
	Tipologija		1.01 Izvirni znanstveni članek

8.Najpomembnejši družbeno-ekonomsko relevantni rezultati projektne skupine[7]

	Družbenoekonomsko relevantni dosežki			
1.	COBISS ID		4737562	Vir: COBISS.SI
	Naslov	*SLO*	Fibrile prionskega proteina aktivirajo NLRP3-inflamasom	
		ANG	NLRP3 inflammasome activation by prion protein fibrils	
	Opis	*SLO*	Pri prionskih boleznih pride do odlaganja patološke oblike PrP v možganih, namnožijo se celice glije in nevroni progresivno odmirajo, vendar pa je vzrok nevrodegeneracije neznan. Obstoječe študije so nakazale vlogo naravne imunosti, predvsem NLRP3-inflamasoma, v drugih amiloidnih boleznih, Alzheimerjevi bolezni in diabetesu tipa 2. Pri različnih tipih prionskih bolezni so opazili izločanje provnetnega citokina IL-1β. Pri miškah z izbitim genom za IL-1R se prionska bolezen razvije kasneje, kar nakazuje na pomembno vlogo IL-1β in s tem tudi inflamasomske poti pri prionskih boleznih. Nas je zanimalo ali lahko odložki prionskega proteina aktivirajo NLRP3-inflamasom in kakšen je mehanizem aktivacije. Pripravili in biofizikalno okarakterizirali smo različne skupke prionskega proteina. Pokazali smo, da prionske fibrile, ne pa nativni monomerni protein ter oligomeri, povzročijo procesiranje pro-IL-1β. S celičnimi linijami z izbitimi geni za posamezne komponente inflamasoma NLRP3, ASC and kaspazo-1, smo pokazali, da pri aktivaciji IL-1β sodeluje NLRP3-inflamasom. K sestavljanju inflamasomskega kompleksa prispevajo različne poti, saj lahko izločanje IL-1β zmanjšajo inhibitorji znižanja K+ v celici in inhibitorji fagocitoze. Fibrile inducirajo mikroglialne celice, da tvorijo nevrotoksične zvrsti in lahko na ta način prispevajo k smrti nevronov. To delo je bilo predstavljeno kot poster na mednarodni konferenci in kot kratko predavanje na konferenci z mednarodno udeležbo. Znanstveni članek je bil poslan v recenzijo (priložen kot priloga).	
			Transmissible spongiform encephalopathies (prion diseases) are characterized by accumulation of abnormal form of prion protein, spongiform change, gliosis and progressive neuronal cell loss; however, the underlying cause of neurodegeneration is not known. Previous studies have indicated the role of innate immunity, particularly of NLRP3 inflammasome	

		ANG	in other amyloid diseases - Alzheimer's disease and type 2 diabetes. Production of proinflammatory cytokine IL-1b has also been observed in brain in several types of prion disease and IL-1R deficiency significantly prolonged the onset of the disease. The aim of our study was to investigate whether prion protein assemblies can instigate NLRP3 inflammasome. We prepared several types of assemblies of prion protein and biophysically characterized them. We show that PrP fibrils, converted from alpha- to predominantly beta-type conformation trigger clevage of pro-IL-1b, which requires the inflammasome components NLRP3, ASC and caspase-1, whereas the native cellular form of prion protein does not induce activation of IL-1b. Several of the previously proposed NLRP3 inflammasome activation pathways may contribute to prion fibril induced inflammasome activation, since it can be blocked by ROS inhibitors, inhibition of K+ efflux and inhibition of phagocytosis. Proteinase K resistant PrP fibrils, but not monomers, induce release of neurotoxic species by microglial cells and can thus contribute to neuronal cell death. This work was presented as a poster at international conference and as a short talk at conference with international participation. Scientific paper was submitted (in attachment).
	Šifra		B.03 Referat na mednarodni znanstveni konferenci
	Objavljeno v		Universty of Verona; Decoding innate immunity, Riva del Garda, Italy, May 4-7, 2011; 2011; Str. 83; Avtorji / Authors: Hafner Bratkovič Iva, Panter Gabriela, Manček Keber Mateja, Fitzgerald Katherine A., Golenbock Douglas, Jerala Roman
	Tipologija		1.12 Objavljeni povzetek znanstvenega prispevka na konferenci
2.	COBISS ID		4382234 Vir: COBISS.SI
	Naslov	*SLO*	Polipeptidni material s prilagodljivimi lastnostmi por
		ANG	Polypeptide material with adjustable pore properties
	Opis	*SLO*	Pripravili smo bionanomaterial s prilagodljivimi lastnostmi por, ki nastane s samosestavljanjem fuzijskih proteinov, sestavljenih iz vsaj dveh proteinskih domen. Ta material se lahko uporabi za ločevanje molekul na osnovi njihovih lastnosti in za kemijsko ali encimsko katalizo.
		ANG	We prepared a polypeptide material with adaptable pore properties, which self-assembles from fusion proteins composed of at least two protein domains. This material can be used for separation of molecules on the basis of their properties and for chemical or enzyme catalysis.
	Šifra		F.33 Patent v Sloveniji
	Objavljeno v		Urad Republike Slovenije za intelektualno lastnino; 2011; 72 str.; Avtorji / Authors: Jerala Roman, Fekonja Ota, Pohar Jelka, Gradišar Helena, Benčina Mojca, Hafner Bratkovič Iva, Bremšak Robert, Miklavčič Špela, Jelerčič Urška, Lukan Anja, Doles Tibor, Božič Sabina, Verce Marko, Debeljak Nika
	Tipologija		2.24 Patent
3.	COBISS ID		4381210 Vir: COBISS.SI
	Naslov	*SLO*	Samosestavljive strukture iz elementov, ki tvorijo obvite vijačnice
		ANG	Self-assembled structures composed of coiled-coil elements
	Opis	*SLO*	Na inovativen način smo pripravili samosestavljive nanostrukture, sestavljene iz polipeptidov, ki tvorijo obvite vijačnice. Na ta način lahko pripravimo nanokletke, nanomreže in nanocevke.
		ANG	In innovative way we prepared self-assembling nanostructures, composed of polypeptides, which are able to form coiled-coils. In this way we are able to prepare nano-cages, nano-nets or nano- tubes.
	Šifra		F.33 Patent v Sloveniji

	Objavljeno v		Urad Republike Slovenije za intelektualno lastnino; 2011; 47 str.; Avtorji / Authors: Jerala Roman, Fekonja Ota, Pohar Jelka, Gradišar Helena, Benčina Mojca, Hafner Bratkovič Iva, Bremšak Robert, Miklavčič Špela, Jelerčič Urška, Lukan Anja, Doles Tibor, Božič Sabina, Verce Marko, Debeljak Nika, Friedrich Jožica	
	Tipologija		2.24 Patent	
4.	COBISS ID		4630298	Vir: COBISS.SI
	Naslov	*SLO*	Nanomateriali na osnovi DNA	
		ANG	DNA-based nanomaterials	
	Opis	*SLO*	S tehniko DNA-origami lahko sestavimo raznovrstne oblike in sicer tako, da dolgo enoverižno molekulo DNA povežemo z velikim številom kratkih verig. S tem, ko smo načrtali in opazili DNA-origamije Slovenije, smo pokazali, da lahko s to tehniko sestavimo tudi nepravilne, asimetrične oblike. Dijaška raziskovalna naloga, ki vključuje te rezultate, je na državnem tekmovanju dosegla zlato priznanje. S sestavljanjem NanoSlovenije smo preverili enostavnost tehnike DNA-origami. V sklopu študentskega tekmovanja Biomod 2011, kjer smo tudi dosegli lepe uspehe, smo pokazali, da lahko s pomočjo specifičnih interakcij na specifičnih pozicijah DNA origami obogatimo z različnimi proteinskimi molekulami. Delo nadaljujemo v smeri aplikacij na področjih materiali in medicina.	
		ANG	DNA origami technique is able to produce a variety of different shapes by constraining a long single stranded DNA molecule with a large number of short oligonucleotides. By designing and observing DNA origamis of Slovenia, we demonstrated that DNA origami technique can be used for construction of irregular asymmetric shapes. High school student research project including these results was awarded gold prize at the national competition of young researchers. With assembly of NanoSlovenia we proved the feasibility of this technique. Taking part in international student competition Biomod 2011, where we also received several prizes, we showed it is possible to enrich DNA origami at specific positions with different protein molecules. Currently we are exploring further applications in material science and medicine.	
	Šifra		D.10 Pedagoško delo	
	Objavljeno v		Slovensko kemijsko društvo =Slovenian Chemical Society; Acta chimica slovenica; 2011; no. 1, Vol. 58; str. 181-184; Impact Factor: 1.011;Srednja vrednost revije / Medium Category Impact Factor: 2.761; WoS: DY; Avtorji / Authors: Jerala Miha, Jerala Roman, Hafner Bratkovič Iva* * corresponding author	
	Tipologija		1.01 Izvirni znanstveni članek	
5.	COBISS ID		4381978	Vir: COBISS.SI
	Naslov	*SLO*	Polipeptidni material, sestavljen iz elastinu podobnih segmentov in segmentov za tvorbo ovitih vijačnic	
		ANG	Polypeptide material composed of elastin-like segments and coiled coil segments	
	Opis	*SLO*	Opisujemo načrtovanje in pripravo polipeptidnega materiala, sestavljenega iz elastinu podobnega segmenta, segmenta, ki tvori obvite vijačnice, in opcijsko funkcionalnih polipeptidnih domen. Možna uporaba teh materialov je za izboljšanje gojenja celic, tkiv in organov, diferenciacije celic, inhibicija rasti patogenov, tretiranje človeških in živalskih tkiv ter kot medicinski oziroma farmacevtski material za uporabo in vivo.	
			We describe the design and preparation of a polypeptide material composed of elastin-like segments, coiled-coil-forming segments and optionally also functional polypeptide domains. Possible applications of these materials are in enhancement of the cell, tissue and organ growth,	

		ANG	for differentiation of cells, inhibition of reproduction of pathogens, treatment of living human or animal tissues and as a medical and pharmaceutical material which will be applicable for treatment of living tissue.
	Šifra		F.33 Patent v Sloveniji
	Objavljeno v		Urad Republike Slovenije za intelektualno lastnino; 2011; 68 str.; Avtorji / Authors: Jerala Roman, Fekonja Ota, Pohar Jelka, Gradišar Helena, Benčina Mojca, Hafner Bratkovič Iva, Bremšak Robert, Miklavčič Špela, Jelerčič Urška, Lukan Anja, Doles Tibor, Božič Sabina, Verce Marko, Debeljak Nika
	Tipologija		2.24 Patent

9.Drugi pomembni rezultati projetne skupine[8]

Vodja podoktorskega projekta redno sodeluje pri izobraževanju mladih kadrov: praktikantov, diplomantov, doktorskih študentov in študentov, ki se z raziskovalnim projektom udeležijo mednarodnih tekmovanj iz sintezne biologije (iGEM) ter v načrtovanju biomolekul (BIOMOD), kjer so študentje dosegli lepe uspehe.

10.Pomen raziskovalnih rezultatov projektne skupine[9]

10.1.Pomen za razvoj znanosti[10]

SLO

Pomen znotrajceličnih receptorjev naravne imunosti, predvsem NLRP3, se povečuje, saj najnovejše raziskave odkrivajo njihovo vlogo pri odzivu proti patogenom in vpletenost v pogostih človeških boleznih. To kaže tudi porast publikacij v najbolj odmevnih revijah, kot so Science, Nature in Molecular Cell. Vendar pa mehanizem aktivacije NLRP3 še ni zadovoljivo pojasnjen. Glavni prispevek naše raziskave je raziskovanje in določitev strukturnih determinant za aktivacijo inflamasoma. Tako smo na osnovi istega monomera - prionskega proteina pripravili in karakterizirali različne različne supramolekularne oblike – od monomera do urejenih in neurejenih agregatov različnih velikosti in pokazali, kakšne so značilnosti aktivatorjev NLRP3-inflamasoma. Naš interdisciplinaren pristop, ki združuje znanje o strukturni in celični biologiji s področjem materialov, predstavlja inovativen in originalen prispevek na teh zelo kompetitivnih področjih.

ANG

Intracellular innate immunity receptors, especially NLRP3, are gaining importance due to their involvement in host pathogen response and in variety of common human pathological conditions, which is also acknowledged by most recent increase of publications in top impact journals, such as Science, Nature and Molecular Cell. Yet, the mechanism of NLRP3 activation has not been explained satisfactorily. The key goal of our research was to investigate and clarify the structural requirements for the activation of inflammasome. Using the same monomer (PrP) we prepared and characterized different supramolecular forms – from monomeric to unordered and ordered aggregates of various size and described the characteristics of NLRP3 inflammasome activators. Our interdisciplinary approach combining the expertise form structural and cell biology as well as material science represents innovative and original contribution in these very competitive disciplines.

10.2.Pomen za razvoj Slovenije[11]

SLO

Interdisciplinarna narava projekta odpira nove možnosti za mednarodno sodelovanje z novimi partnerji ali pa z raziskovalci, s katerimi smo že sodelovali znotraj evropskih projektov ali bilateralnih sodelovanj. Naš laboratorij je že prispeval nekaj pomembnih odkritij na področju naravne imunosti, predvsem v povezavi z membranskimi Toll-u podobnimi receptorji. Ta projekt razširja raziskave naravne imunosti k določanju aktivacijskih mehanizmov znotrajceličnih

receptorjev.

ANG

The interdisciplinary nature of the project opens new possibilities for international cooperation with researchers with whom we already collaborated e.g. within European projects and bilateral projects as well with new collaborators.
Our department made some important contributions to the field of innate immunity, particularly in connection to membrane Toll-like receptors. This project is expanding the research of innate immunity towards defining the mechanisms of intracellular receptors.

11. Samo za aplikativne projekte!
Označite, katerega od navedenih ciljev ste si zastavili pri aplikativnem projektu, katere konkretne rezultate ste dosegli in v kakšni meri so doseženi rezultati uporabljeni

Cilj		
F.01	**Pridobitev novih praktičnih znanj, informacij in veščin**	
	Zastavljen cilj	○ DA ○ NE
	Rezultat	
	Uporaba rezultatov	
F.02	**Pridobitev novih znanstvenih spoznanj**	
	Zastavljen cilj	○ DA ○ NE
	Rezultat	
	Uporaba rezultatov	
F.03	**Večja usposobljenost raziskovalno-razvojnega osebja**	
	Zastavljen cilj	○ DA ○ NE
	Rezultat	
	Uporaba rezultatov	
F.04	**Dvig tehnološke ravni**	
	Zastavljen cilj	○ DA ○ NE
	Rezultat	
	Uporaba rezultatov	
F.05	**Sposobnost za začetek novega tehnološkega razvoja**	
	Zastavljen cilj	○ DA ○ NE
	Rezultat	
	Uporaba rezultatov	
F.06	**Razvoj novega izdelka**	
	Zastavljen cilj	○ DA ○ NE
	Rezultat	
	Uporaba rezultatov	
F.07	**Izboljšanje obstoječega izdelka**	
	Zastavljen cilj	○ DA ○ NE
	Rezultat	
	Uporaba rezultatov	

F.08	**Razvoj in izdelava prototipa**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.09	**Razvoj novega tehnološkega procesa oz. tehnologije**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.10	**Izboljšanje obstoječega tehnološkega procesa oz. tehnologije**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.11	**Razvoj nove storitve**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.12	**Izboljšanje obstoječe storitve**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.13	**Razvoj novih proizvodnih metod in instrumentov oz. proizvodnih procesov**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.14	**Izboljšanje obstoječih proizvodnih metod in instrumentov oz. proizvodnih procesov**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.15	**Razvoj novega informacijskega sistema/podatkovnih baz**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.16	**Izboljšanje obstoječega informacijskega sistema/podatkovnih baz**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.17	**Prenos obstoječih tehnologij, znanj, metod in postopkov v prakso**	
	Zastavljen cilj	DA NE

	Rezultat	
	Uporaba rezultatov	
F.18	**Posredovanje novih znanj neposrednim uporabnikom (seminarji, forumi, konference)**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.19	**Znanje, ki vodi k ustanovitvi novega podjetja ("spin off")**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.20	**Ustanovitev novega podjetja ("spin off")**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.21	**Razvoj novih zdravstvenih/diagnostičnih metod/postopkov**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.22	**Izboljšanje obstoječih zdravstvenih/diagnostičnih metod/postopkov**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.23	**Razvoj novih sistemskih, normativnih, programskih in metodoloških rešitev**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.24	**Izboljšanje obstoječih sistemskih, normativnih, programskih in metodoloških rešitev**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.25	**Razvoj novih organizacijskih in upravljavskih rešitev**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.26	**Izboljšanje obstoječih organizacijskih in upravljavskih rešitev**	
	Zastavljen cilj	DA NE
	Rezultat	

	Uporaba rezultatov	
F.27	**Prispevek k ohranjanju/varovanje naravne in kulturne dediščine**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.28	**Priprava/organizacija razstave**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.29	**Prispevek k razvoju nacionalne kulturne identitete**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.30	**Strokovna ocena stanja**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.31	**Razvoj standardov**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.32	**Mednarodni patent**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.33	**Patent v Sloveniji**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.34	**Svetovalna dejavnost**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	
F.35	**Drugo**	
	Zastavljen cilj	DA NE
	Rezultat	
	Uporaba rezultatov	

Komentar

12.Samo za aplikativne projekte!
Označite potencialne vplive oziroma učinke vaših rezultatov na navedena področja

	Vpliv	Ni vpliva	Majhen vpliv	Srednji vpliv	Velik vpliv	
G.01	**Razvoj visoko-šolskega izobraževanja**					
G.01.01.	Razvoj dodiplomskega izobraževanja	○	○	○	○	
G.01.02.	Razvoj podiplomskega izobraževanja	○	○	○	○	
G.01.03.	Drugo:	○	○	○	○	
G.02	**Gospodarski razvoj**					
G.02.01	Razširitev ponudbe novih izdelkov/storitev na trgu	○	○	○	○	
G.02.02.	Širitev obstoječih trgov	○	○	○	○	
G.02.03.	Znižanje stroškov proizvodnje	○	○	○	○	
G.02.04.	Zmanjšanje porabe materialov in energije	○	○	○	○	
G.02.05.	Razširitev področja dejavnosti	○	○	○	○	
G.02.06.	Večja konkurenčna sposobnost	○	○	○	○	
G.02.07.	Večji delež izvoza	○	○	○	○	
G.02.08.	Povečanje dobička	○	○	○	○	
G.02.09.	Nova delovna mesta	○	○	○	○	
G.02.10.	Dvig izobrazbene strukture zaposlenih	○	○	○	○	
G.02.11.	Nov investicijski zagon	○	○	○	○	
G.02.12.	Drugo:	○	○	○	○	
G.03	**Tehnološki razvoj**					
G.03.01.	Tehnološka razširitev/posodobitev dejavnosti	○	○	○	○	
G.03.02.	Tehnološko prestrukturiranje dejavnosti	○	○	○	○	
G.03.03.	Uvajanje novih tehnologij	○	○	○	○	
G.03.04.	Drugo:	○	○	○	○	
G.04	**Družbeni razvoj**					
G.04.01	Dvig kvalitete življenja	○	○	○	○	
G.04.02.	Izboljšanje vodenja in upravljanja	○	○	○	○	
G.04.03.	Izboljšanje delovanja administracije in javne uprave	○	○	○	○	
G.04.04.	Razvoj socialnih dejavnosti	○	○	○	○	
G.04.05.	Razvoj civilne družbe	○	○	○	○	
G.04.06.	Drugo:	○	○	○	○	
G.05.	**Ohranjanje in razvoj nacionalne naravne in kulturne dediščine in identitete**	○	○	○	○	
G.06.	**Varovanje okolja in trajnostni razvoj**	○	○	○	○	

G.07	**Razvoj družbene infrastrukture**					
G.07.01.	Informacijsko-komunikacijska infrastruktura	○	○	○	○	
G.07.02.	Prometna infrastruktura	○	○	○	○	
G.07.03.	Energetska infrastruktura	○	○	○	○	
G.07.04.	Drugo:	○	○	○	○	
G.08.	**Varovanje zdravja in razvoj zdravstvenega varstva**	○	○	○	○	
G.09.	**Drugo:**	○	○	○	○	

Komentar

13.Pomen raziskovanja za sofinancerje[12]

	Sofinancer			
1.	Naziv			
	Naslov			
	Vrednost sofinanciranja za celotno obdobje trajanja projekta je znašala:			EUR
	Odstotek od utemeljenih stroškov projekta:			%
	Najpomembnejši rezultati raziskovanja za sofinancerja			Šifra
	1.			
	2.			
	3.			
	4.			
	5.			
	Komentar			
	Ocena			

C. IZJAVE

Podpisani izjavljam/o, da:

- so vsi podatki, ki jih navajamo v poročilu, resnični in točni
- se strinjamo z obdelavo podatkov v skladu z zakonodajo o varstvu osebnih podatkov za potrebe ocenjevanja ter obdelavo teh podatkov za evidence ARRS
- so vsi podatki v obrazcu v elektronski obliki identični podatkom v obrazcu v pisni obliki
- so z vsebino zaključnega poročila seznanjeni in se strinjajo vsi soizvajalci projekta

Podpisi:

zastopnik oz. pooblaščena oseba raziskovalne organizacije: in *vodja raziskovalnega projekta:*

Kemijski inštitut Iva Hafner Bratkovič

ŽIG

Kraj in datum:	Ljubljana	13.3.2012

Oznaka prijave: ARRS-RPROJ-ZP-2012/18

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[1] Zaradi spremembe klasifikacije je potrebno v poročilu opredeliti raziskovalno področje po novi klasifikaciji FOS 2007 (Fields of Science). Prevajalna tabela med raziskovalnimi področji po klasifikaciji ARRS ter po klasifikaciji FOS 2007 (Fields of Science) s kategorijami WOS (Web of Science) kot podpodročji je dostopna na spletni strani agencije (http://www.arrs.gov.si/sl/gradivo/sifranti/preslik-vpp-fos-wos.asp). Nazaj

[2] Napišite povzetek raziskovalnega projekta (največ 3.000 znakov v slovenskem in angleškem jeziku) Nazaj

[3] Napišite kratko vsebinsko poročilo, kjer boste predstavili raziskovalno hipotezo in opis raziskovanja. Navedite ključne ugotovitve, znanstvena spoznanja, rezultate in učinke raziskovalnega projekta in njihovo uporabo ter sodelovanje s tujimi partnerji. Največ 12.000 znakov vključno s presledki (približno dve strani, velikosti pisave 11). Nazaj

[4] Realizacija raziskovalne hipoteze. Največ 3.000 znakov vključno s presledki (približno pol strani, velikosti pisave 11) Nazaj

[5] V primeru bistvenih odstopanj in sprememb od predvidenega programa raziskovalnega projekta, kot je bil zapisan v predlogu raziskovalnega projekta oziroma v primeru sprememb, povečanja ali zmanjšanja sestave projektne skupine v zadnjem letu izvajanja projekta (obrazložitev). V primeru, da sprememb ni bilo, to navedite. Največ 6.000 znakov vključno s presledki (približno ena stran, velikosti pisave 11). Nazaj

[6] Znanstveni in družbeno-ekonomski dosežki v programu in projektu so lahko enaki, saj se projekna vsebina praviloma nanaša na širšo problematiko raziskovalnega programa, zato pričakujemo, da bo večina izjemnih dosežkov raziskovalnih programov dokumentirana tudi med izjemnimi dosežki različnih raziskovalnih projektov.

Raziskovalni dosežek iz obdobja izvajanja projekta (do oddaje zaključnega poročila) vpišete tako, da izpolnite COBISS kodo dosežka – sistem nato sam izpolni naslov objave, naziv, IF in srednjo vrednost revije, naziv FOS področja ter podatek, ali je dosežek uvrščen v A'' ali A'. Nazaj

[7] Znanstveni in družbeno-ekonomski dosežki v programu in projektu so lahko enaki, saj se projekna vsebina praviloma nanaša na širšo problematiko raziskovalnega programa, zato pričakujemo, da bo večina izjemnih dosežkov raziskovalnih programov dokumentirana tudi med izjemnimi dosežki različnih raziskovalnih projektov.

Družbeno-ekonomski rezultat iz obdobja izvajanja projekta (do oddaje zaključnega poročila) vpišete tako, da izpolnite COBISS kodo dosežka – sistem nato sam izpolni naslov objave, naziv, IF in srednjo vrednost revije, naziv FOS področja ter podatek, ali je dosežek uvrščen v A'' ali A'.

Družbenoekonomski dosežek je po svoji strukturi drugačen, kot znanstveni dosežek. Povzetek znanstvenega dosežka je praviloma povzetek bibliografske enote (članka, knjige), v kateri je dosežek objavljen.

Povzetek družbeno ekonomsko relevantnega dosežka praviloma ni povzetek bibliografske enote, ki ta dosežek dokumentira, ker je dosežek sklop več rezultatov raziskovanja, ki je lahko dokumentiran v različnih bibliografskih enotah. COBISS ID zato ni enoznačen izjemoma pa ga lahko tudi ni (npr. v preteklem letu vodja meni, da je izjemen dosežek to, da sta se dva mlajša sodelavca zaposlila v gospodarstvu na pomembnih raziskovalnih nalogah, ali ustanovila svoje podjetje, ki je rezultat prejšnjega dela … - v obeh primerih ni COBISS ID). Nazaj

[8] Navedite rezultate raziskovalnega projekta iz obdobja izvajanja projekta (do oddaje zaključnega poročila) v primeru, da katerega od rezultatov ni mogoče navesti v točkah 7 in 8 (npr. ker se ga v sistemu COBISS ne vodi). Največ 2.000 znakov vključno s presledki. Nazaj

[9] Pomen raziskovalnih rezultatov za razvoj znanosti in za razvoj Slovenije bo objavljen na spletni strani: http://sicris.izum.si/ za posamezen projekt, ki je predmet poročanja Nazaj

[10] Največ 4.000 znakov vključno s presledki Nazaj

[11] Največ 4.000 znakov vključno s presledki Nazaj

[12] Rubrike izpolnite / prepišite skladno z obrazcem "izjava sofinancerja" http://www.arrs.gov.si/sl/progproj/rproj/gradivo/, ki ga mora izpolniti sofinancer. Podpisan obrazec "Izjava sofinancerja" pridobi in hrani nosilna raziskovalna organizacija – izvajalka projekta. Nazaj

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NLRP3 activation by prion protein fibrils as the source of IL-1β, neuronal toxicity and microglial priming

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ABBREVIATIONS: PrP - prion protein**, NLRP3** - NACHT, LRR and PYD domains-containing protein 3**, ASC** - apoptosis-associated speck-like protein

ABSTRACT

Prion diseases are fatal transmissible neurodegenerative diseases, characterized by aggregation of the pathological form of prion protein, spongiform degeneration, neuronal loss and activation of astrocytes and microglia. Microglia can clear prion plaques but on the other hand cause neuronal death via release of neurotoxic species. Elevated expression of the proinflammatory cytokine IL-1β has been observed in brains affected by several prion diseases and IL-1R-deficiency significantly prolonged the onset of the neurodegeneration in mice. We show that microglia and macrophages release IL-1β upon stimulation by PrP fibrils, which depends on the NLRP3 inflammasome. Activation of NLRP3 inflammasome by PrP fibrils requires depletion of intracellular K^+ and requires phagocytosis of PrP fibrils and consecutive lysosome destabilization. Among the well-defined molecular forms of PrP the strongest NLRP3 activation was observed by fibrils, followed by aggregates, while neither native monomeric nor oligomeric PrP were able to activate the NLRP3 inflammasome. We propose that NLRP3 inflammasome activation by PrP fibrils could induce the primed state of microglia, which makes the brain tissue hyper-responsive to the activation of the NF-κB inducing signal. Our results together with previous studies on IL-1R-deficient mice suggest the IL-1 signaling pathway as the perspective target for the therapy of prion disease.

INTRODUCTION

Prion diseases are lethal neurodegenerative diseases affecting human and other mammalian species. These diseases include scrapie in sheep, bovine spongiform encephalopathy in cow and chronic wasting disease in deer and elk. Transmission of scrapie to mice led to development of many experimental prion strains differing in incubation period and profiles of pathological lesions in the central nervous system. Human prion diseases can be acquired, sporadic or genetic. Creutzfeld-Jakob disease (CJD) is the most common type with incidence about one per million per year. Hereditary forms include familial CJD, fatal familial insomnia

and Gerstmann-Sträussler-Scheinker syndrome, and originate from mutations in the gene encoding the prion protein. Acquired human prion diseases are kuru, new variant CJD (due to ingestion of contaminated beef) and iatrogenic forms. Prion diseases are enigmatic since the infectious agent is composed primarily of an abnormal form of prion protein, PrP^{Sc}(Prusiner, 1998). During prion disease normal prion protein PrP^{c} converts to protease-resistant PrP^{Sc}. The three-dimensional structure of PrP^{Sc} has not been resolved, but is in contrast to the native predominantly α-helical PrP^{C}, enriched in β-secondary structure and forms amyloid fibrils. The major characteristics of prion diseases revealed by *post mortem* analysis of human brain sections are spongiform degeneration of neurons, neuronal loss, intense reactive astrocytosis and accumulation of amyloid plaques composed of PrP^{Sc} (Budka et al., 1995). Analysis of brains of scrapie-infected mice also revealed recruitment and activation of microglia, which are associated with PrP^{Sc} deposits (Betmouni et al., 1996; Williams et al., 1994). Activation of microglia precedes the onset of the disease and coincides with increased deposition of PrP^{Sc} (Giese et al., 1998). Activated astrocytes and microglia release proinflammatory cytokines and neurotoxic agents such as NO, free radicals and glutamate (Suzumura et al., 2006). The proinflammatory cytokine IL-1β mRNA is upregulated more than 20-fold in the prion-infected microglia in comparison to non-infected control (Baker and Manuelidis, 2003). IL-1β is also released from astrocytes exposed to infectious brain homogenate (Tribouillard-Tanvier et al., 2009). The incubation period upon infection with two different prion strains is increased in IL-1R-deficient mice, suggesting that IL-1 plays an important role in the disease progression (Schultz et al., 2004; Tamguney et al., 2008). Interestingly, in ME7 prion strain the expression of IL-1β is low, but is exacerbated upon LPS activation (Combrinck et al., 2002). Combrinck and co-workers propose that in chronic inflammatory neurodegenerative diseases microglia are in the primed state and respond to peripheral infection by increased IL-1β production (Combrinck et al., 2002) and increased neuronal death (Cunningham et al., 2005).

Pro-IL-1β transcription is initiated upon NF-κB activation, however, the second trigger is required for the release of IL-1β. The active form IL-1β is produced by proteolysis of its precursor form by an intracellular protein complex called the inflammasome (Petrilli et al., 2007). The most investigated NLRP3 inflammasome is composed of a sensor NLRP3, an adaptor ASC and a pro-caspase-1. Upon activation, inflammasomes self-assemble which leads to the autoactivation of pro-caspase-1. The active caspase-1 in turn cleaves and activates pro-IL-1β. Very different molecular instigators have been shown to activate NLRP3 inflammasome: ATP, microbial pore-forming toxins (Gurcel et al., 2006; Mariathasan et al., 2006), serum amyloid A (Niemi et al., 2011), schistosomal egg antigens (Ritter et al., 2010), but also aggregates such as uric acid crystals (Martinon et al., 2006), silica (Dostert et al., 2008; Hornung et al., 2008), cholesterol crystals (Duewell et al., 2010) and asbestos fibres (Dostert et al., 2008).

Halle and co-workers showed that the NLRP3 inflammasome is triggered by aggregated Aβ(1-42) , which is the main component of senile plaques in Alzheimer's disease (Halle et al., 2008). This motivated us to explore if and which forms of PrP could activate the NLRP3 inflammasome. We demonstrate that fibrils induce NF-κB and thus provide the priming signal for the production of the pro-IL-1β and NLRP3. We also show that PrP fibrils trigger NLRP3 inflammasome assembly, which can be inhibited by inhibitors of K^+ efflux and phagocytosis. We show that PrP fibrils are the most potent activator of the NLRP3 inflammasome, whereas oligomers and monomeric native forms do not activate inflammasomes at all. We also show that activated microglia contribute to neuronal death and suggest that in prion diseases the primed state of microglia actually occurs as a consequence of the activation of the NLRP3 inflammasome, making the microglia and brain tissue sensitive to the inflammatory signals that lead to activation of NF-κB and production of pro-IL-1β.

RESULTS

PrP fibril-activated microglia are neurotoxic

One of the major characteristics of prion diseases is reactive gliosis. Activated microglial cells gather around prion plaques in diseased brain tissue (Betmouni et al., 1996; Williams et al., 1994). PrP^{Sc} was also found in microglial cells (Andreoletti et al., 2002). While fibrils by themselves can be toxic to neurons (Novitskaya et al., 2006), we wanted to assess whether the fibril toxicity is increased upon activation of microglial cells. Neurons were cultured either alone (Fig. 1A, upper row) or in the co-culture with microglia (Fig. 1A, bottom row) and primed with LPS (Fig. 1A, left) or primed with LPS and activated with PrP fibrils (Fig. 1A, right). Interestingly, we did not observe any direct fibril toxicity to neurons at used concentrations (Fig. 1A, Supplementary fig. 1), while the number of neurons decreased when the co-culture was stimulated with PrP fibrils (Fig. 1A, Supplementary fig. 1). We also show that microglial cells upon priming with IFN-γ and activation with synthetic PrP105-125 fibrils release free radical NO similar to treatment with LPS or zymosan (Fig. 1B). This demonstrates that PrP fibrils are able to activate microglia to release neurotoxic species, such as NO, which induces neuronal death.

IL-1β is released from macrophages and microglia activated by PrP fibrils

Since IL-1β is one of the major proinflammatory cytokines upregulated in prion disease (Baker and Manuelidis, 2003), we investigated whether PrP fibrils are able to induce IL-1β secretion. Release of IL-1β by LPS-primed macrophages and microglia into the cell culture medium was monitored, demonstrating that PrP fibrils are indeed able to induce IL-1β production (Fig.2 A, B). We detected mature IL-1β (p17) in cell culture medium of activated macrophages by Western blotting, while no IL-1β was processed from unprimed or primed only macrophages (Fig. 2C). As previously observed for maturation of IL-1β by uric acid crystals (Martinon et al., 2006), the amount of pro-IL-1β in the cell decreases with increased release of the active form (Fig. 2C). To ensure strong activation of NF-κB and thus expression of pro-IL-1β cells were primed with LPS. In our experimental setup we observed no IL-1β release due to PrP fibrils in the absence of priming (Fig. 2 A, B). Nevertheless, several studies have shown that amyloid fibrils are able to induce NF-κB (Bacot et al., 2003;

Spinner et al., 2008; Stewart et al., 2010). To test whether the NF-κB priming could be facilitated through PrP fibrils, we subjected indicator RAW-Blue cell line to synthetic LPS-free PrP105-125 fibrils. RAW-Blue cell line expresses NF-κB/AP-1-inducible secreted embryonic alkaline phosphatase. Indeed, PrP105-125 fibrils induced concentration-dependent secretion of alkaline phosphatase. This shows that PrP fibrils are able to induce IL-1β release, but can also provide the signal for NF-κB activation, necessary for expression of pro-IL-1β and NLRP3.

PrP fibrils activate caspase-1

Caspase-1 is the main activator of IL-1β maturation in mononuclear phagocytes. To follow the activation of caspase-1, we used fluorescent covalent inhibitor, which binds only to active caspase-1 but not to procaspase-1. We analyzed the activation of caspase-1 in primed and PrP fibril-activated macrophages as detected by confocal microscopy (Fig. 3A) and flow cytometry (Fig. 3B). Active caspase-1 subunit p10 was also detected in the cell culture medium upon activation by fibrils and in a positive control, stimulated by ATP by Western blot (Fig. 3C). There was no caspase-1 activation in unprimed or LPS primed only cells (Fig. 3C). Further, immortalized microglial cells from the caspase-1-deficient; IL-1R-deficient and matching wild-type mice were treated with PrP fibrils. Caspase-1-deficient microglia failed to secrete IL-1β while wild-type microglia dose-dependently released IL-1β (Fig. 3D). IL-1R-deficient microglia efficiently released IL-1β, demonstrating that PrP fibril-induced secretion of IL-1β is independent of IL-1R signaling (Fig. 3D). Additionally, caspase-1 inhibitor Z-YVAD-FMK inhibits maturation of IL-1β (Fig. 3E). We thereby demonstrated that PrP fibrils induced maturation of IL-1β in a manner that depends on caspase-1 activity.

PrP fibrils activate NLRP3 inflammasome

Caspase-1 is activated by several distinct NLR or PYHIN containing inflammasomes. Since several other particulate triggers (Dostert et al., 2008; Halle et al., 2008; Hornung et al., 2008; Martinon et al., 2006) were shown to activate the NLRP3 inflammasome, we wanted to

investigate if the same mechanism is also responsible for activation of caspase-1 by aggregated PrP, the hallmark of prion diseases. We show that production of IL-1β induced by PrP fibrils is abrogated in macrophages deficient in NLRP3 or ASC, while wild-type macrophages are efficiently and dose-dependently activated by PrP fibrils (Fig. 4A). The inability of macrophages deficient in NLRP3 or the signaling adaptor ASC to release IL-1β demonstrates that PrP fibrils induce activation of the NLRP3 inflammasome, which in turn activates caspase-1 and IL-1β.

Further we investigated the mechanism of NLRP3 inflammasome activation by PrP fibrils. PrP fibrils could activate NLRP3 directly or indirectly through induction of other activators of the NLRP3 inflammasome. Since PrP fibrils are able to induce cell death in macrophages (Supplementary fig. 2), it is possible that dying cells could release ATP, which is a potent activator of the NLRP3 inflammasome. This is not the case, since the addition of apyrase, the ATP-hydrolyzing enzyme, had no effect on IL-1β release by PrP fibrils in contrast to the ATP-stimulated control (Fig. 4B). On the other hand depletion of intracellular K^+ has also been described as characteristic for NLRP3 inflammasome activation (Petrilli et al., 2007). Inhibition of the cytoplasmic K^+ efflux either by the addition of KCl-conditioned media or glyburide, an inhibitor of ATP-dependent K^+ channels, decreased the activation of inflammasome by PrP fibrils (Fig. 4C), demonstrating the role of K^+ efflux in the activation.

Phagocytosis of PrP fibrils results in lysosome destabilization

To follow the fibril-macrophage interaction, PrP fibrils were fluorescently labeled, which didn't affect the activation process. After several hours, the majority of macrophages contained internalized PrP fibrils (Fig. 5A). Inhibition of phagocytosis by the addition of cytochalasin D, a phagocytosis inhibitor, which blocks actin polymerization, correlated with decrease of the inflammasome activation (Fig. 5B). This suggests that phagocytosis of PrP fibrils is necessary for the inflammasome activation as has been previously reported for several other particulate activators (Dostert et al., 2008; Duewell et al., 2010; Halle et al., 2008; Hornung et

al., 2008). Unlabelled fibrils were added to macrophages together with DQ-ovalbumin. DQ-ovalbumin exhibits fluorescence only in compartments where proteases degrade ovalbumin resulting in dequenching of the fluorophore. Phagocytosis of PrP fibrils induced lysosome swelling (Fig. 5C right), while in macrophages that were not exposed to fibrils DQ-ovalbumin labeled small vesicles (Fig. 5C left). Similar results were also obtained with fluorescent dextran as an alternative endosomal marker (Supplementary Fig. 3). As in the case of silica (Hornung et al., 2008) and Aβ fibrils (Halle et al., 2008), we also observed cytosolic DQ-ovalbumin staining in PrP fibril-treated macrophages (Fig. 5D), suggesting that ingestion of PrP fibrils led to leakage of the lysosomal content into the cytosol.

Fibrillar form is the most potent inflammasome activating molecular form of the PrP

The requirement for the type of the protein molecular assemblies that can induce NLRP3 inflammasome has so far not been well characterized by parallel testing of several isolated and well-characterized forms. We prepared and characterized several different PrP assemblies: amorphous aggregates, PrP fibrils, PrP oligomers and monomeric PrP. Monomeric PrP has a predominantly α-helical secondary structure (Supplementary Fig. 4A), while PrP fibrils as well as oligomers have an increased content of the β-secondary structure (Supplementary fig. 4B). *In vitro* prepared PrP fibrils and oligomers correspond to PrP species found in the diseased brain. It has been shown recently that infectious prion disease can be induced by *in vitro* converted bacterially expressed PrP (Kim et al., 2010; Legname et al., 2004; Makarava et al., 2010; Wang et al., 2010). Additionally, we also prepared PrP aggregates, which have no equivalent in nature. These PrP aggregates vary in size from as low as 1 μm to almost 100 μm, with most particles in the range of 10-20 μm (Fig. 6A left). We show that PrP aggregates are able to activate the NLRP3 inflammasome (Fig. 6A right). PrP fibrils are approximately 7 nm thin fibrils with lengths in the range from 100 nm to several μm (Fig. 6B left) (Hafner-Bratkovic et al., 2011). We demonstrated throughout this paper that PrP fibrils activate the NLRP3 inflammasome. If we compare the level of secreted IL-1β, PrP fibrils are significantly more efficient in inflammasome activation than PrP aggregates (Fig.

6A and 6B). While the conversion efficiency to PrP fibrils is 100% (Hafner-Bratkovic et al., 2011), the conversion efficiency to oligomers, estimated from gel filtration analysis was ~ 40% (Supplementary fig. 4C). Oligomers measure about 30 nm in diameter as estimated from AFM images (Fig. 6C). Surprisingly, oligomers did not activate the inflammasome (Fig. 6C) and neither did the native non pathological monomeric α-PrP (Fig. 6D). Using different, well characterized PrP assemblies, we were able to show that NLRP3 inflammasome activation via particulate protein assemblies depends on the type of aggregate. We suggest that PrP fibrils are potent activators since they are readily phagocytosed by macrophages, but are resistant to proteolysis and cause lysosome destabilization. Oligomers and monomeric PrP do not cause leakage of lysosomal content into the cytosol and thus do not activate NLRP3 inflammasome.

DISCUSSION

Prion diseases are characterized by neuroinflammation, which contributes to the loss of neurons and decay of the cognitive functions. We have shown that PrP fibrils induce production of IL-1β through activation of the NLRP3 inflammasome. We tested several different *in vitro* prepared PrP forms and showed that the larger particles (aggregates and fibrils) activate the NLRP3 inflammasome in contrast to native α-form. PrP oligomers, which have been proposed as the most infectious PrP species (Silveira et al., 2005), do not activate the inflammasome. Previous studies identified two amyloid activators of NLRP3 inflammasome: Aβ fibrils (Halle et al., 2008) and amylin oligomers (Masters et al., 2010). Masters and co-workers have shown that the NLRP3 inflammasome is activated by amylin oligomeric fractions with molecular weight of less than 100 kDa (Masters et al., 2010). We observed no activation with PrP oligomers, which are even larger than amylin oligomers (Fig. 6C). Filtering through 200 nm filter almost completely abolished the activation by PrP fibrils (not shown), thus the mechanism of inflammasome activation by different amyloids may not

be identical. Some amyloid-forming peptides are prone to aggregation, thus careful physical characterization immediately prior to cell stimulation is required in order to identify the present molecular species. The size dependence of inflammasome activation by PrP assemblies is in accordance with the activation of NLRP3 inflammasome by differently-sized polystyrene microparticles (Sharp et al., 2009).

NLRP3 inflammasome activation by PrP fibrils leads to release of proinflammatory cytokine IL-1β, which signals through IL-1R. Upon activation IL-1R recruits the intracellular adaptor MyD88. Inoculation of MyD88-deficient mice with prion strain RML demonstrated no significant delay in the incubation period (Prinz et al., 2003). However, two independent studies reported prolonged incubation period in IL-1R-deficient mice infected by prion strains 139A and RML (Schultz et al., 2004; Tamguney et al., 2008), suggesting that IL-1β does participate in the pathophysiology of the disease. Schultz and co-workers showed significantly delayed activation of astrocytes and delayed accumulation of the pathological prion protein in IL-1R-deficient mice (Schultz et al., 2004). Microglia is activated by deposited amyloid and this activation is IL-1R-independent (Schultz et al., 2004), which is also supported by our experiments (Fig. 3D). Activated microglia could exert their neurotoxic effects either directly through production of NO (Fig. 1) (Brown et al., 1996) or as proposed by Schultz et al. through activation of astrocytes, which is at least partially dependent on IL-1 (Schultz et al., 2004). Activated astrocytes also secrete neurotoxic factors *in vitro* (Brown, 1999) and are able to replicate prions *in vivo* (Raeber et al., 1997).

There are conflicting reports regarding the production of IL-1β in prion infected brains and particular types of cells (Baker and Manuelidis, 2003; Tribouillard-Tanvier et al., 2009; Walsh et al., 2001). While PrP fibrils are able to activate NF-κB, this activation might not be sufficient to produce prominent amounts of pro-IL-1β, but can induce the primed state of microglia by activation of the NLRP3 inflammasome. IL-1β production in prion infected tissue may be potentially enhanced in case of an additional proinflammatory first signal, which stimulates the production of pro-IL-1β, such as e.g. viral or bacterial infection or endogenous

danger signals. In ME7 mouse prion model stimulation with LPS triggered a robust expression of IL-1β even by systemic i.p. administration (Cunningham et al., 2005), supporting the conclusion that the activation signal, provided by NLRP3 described in this report is responsible for this activated phenotype of microglia. Prion strains differ among others in the type of PrP^{Sc} deposits and the size of the most infectious particles (Tixador et al., 2010), thus it is possible that the role of NLRP3 inflammasome can vary depending on the predominant PrP^{Sc} size.

However, the role of microglia in prion disease as well as in other amyloidoses is not only harmful. Depletion of microglia resulted in increased accumulation of PrP^{Sc} (Falsig et al., 2008), suggesting that microglial cells are able to decrease prion infectivity and progression of the disease by phagocytosis of PrP^{Sc} as has been shown for macrophages in the spleen (Beringue et al., 2000). Administration of complete Freund's adjuvant to prion-infected mice increased incubation period, possibly through activation of phagocytosis (Tal et al., 2003), further supporting the protective role of microglia. Thus the activation of microglia is a double-edged sword, participating both in the degradation of protein amyloids and on the other hand in production of proinflammatory and neurotoxic mediators. This double role of the microglia may explain the apparent discrepancy between the results of MyD88 and IL-1R deficiency. Therefore, the indiscriminatory inhibition of innate immune response in prion diseases is not appropriate. On the other hand specific inhibition of the inflammasome or its products that does not inhibit the phagocytosis or perhaps at the stage when the phagocytic capacity of microglia is already overwhelmed, could be beneficial in therapy of prion disease and other amyloidoses.

MATERIALS AND METHODS

Materials. Z-YVAD-FMK was from Biovision. All other chemicals (if not specified otherwise in the text) were from Sigma.

α-PrP isolation and refolding. Prion protein was produced in bacteria in the form of inclusion bodies and refolded on a Ni^{2+}-NTA column (Quiagen) by gradual decrease of guanidine hydrochloride and β-mercaptoethanol concentration to 0 M as previously described (Avbelj et al., 2011; Gaspersic et al., 2010; Hafner-Bratkovic et al., 2011; Hafner-Bratkovic et al., 2011; Hafner-Bratkovic et al., 2008), with additional step of thorough washing with 60% isopropanol to remove LPS (Franken et al., 2000) before elution with 0.5 M imidazole, pH 5.8. The content of LPS as determined by LAL test (Lonza) was less than 0.15 ng/mg of protein.

Preparation of other forms of prion protein and characterization. The amyloid PrP fibrils were produced by shaking denatured PrP in 1 M guanidine hydrochloride, 3M urea in PBS pH 6.8 at protein concentrations 154 μM at 37°C in a microtiter plate-format (Bocharova et al., 2005; Hafner-Bratkovic et al., 2011) . Formation of fibrils was followed by an amyloid specific dye Thioflavin T. The PrP oligomer form was prepared by diluting protein from 10 M urea to 5 M urea, 0.2 M NaCl, 20 mM sodium acetate buffer, pH 4.0 and incubating overnight at room temperature (Baskakov et al., 2002). To determine the yield of conversion, conversion reaction was analyzed by gel filtration on biosep-sec-s 4000 (Phenomenex) column (Hafner-Bratkovic et al., 2008). Precipitate after dialysis against MilliQ water or dialyzed methanol-precipitated PrP served as amorphous PrP aggregates. Precipitates were observed under the confocal microscope (bright field) Leica TCS SP5. Prior to characterization and cell culture assays, fibrils and oligomers were dialyzed against PBS. PrP105-125 fibrils were prepared by dissolving synthetic peptide (Keck Biotechnology) in LPS-free PBS and shaking overnight at 37°C. Formation of fibrils was followed by Thioflavin T.

Circular dichroism. Far-UV CD spectra were recorded on an Applied Photophysics Chirascan spectropolarimeter between 190 and 250 nm in a 1 mm path length cuvette at a protein concentration of 0.1 mg/ml.

Atomic force microscopy. To observe fibrils, oligomers and monomeric α-PrP by atomic force microscopy a drop of protein sample (diluted to 0.22 μM) was applied to freshly cleaved mica (Ted Pella) and left to adsorb for 5 min after which it was washed twice with filtered Milli-Q water and dried under nitrogen. Samples were observed by Agilent Technologies 5500 Scanning Probe Microscope operating in acoustic alternating current mode utilizing silicon cantilevers (Arrow-NCR, NanoWorld) (Hafner-Bratkovic et al., 2011; Hafner-Bratkovic et al., 2011).

Cell cultures. Preparation of immortalized microglial cells from caspase-1-deficient and IL-1R-deficient mice and macrophages from NLRP3-deficient mice and ASC-deficient mice and corresponding wild-type control (C57BL/6) was described by Halle et al. (Halle et al., 2008) and Hornung et al. (Hornung et al., 2008). Immortalized microglial cells, immortalized macrophages and RAW-Blue cells were cultured in DMEM supplemented with 10% FBS. CAD cell line (Qi et al., 1997) was cultured in Optimem medium supplemented with 10% FBS. All cell culture supplies were from Invitrogen.

IL-1β ELISA. All experiments were performed in serum-free DMEM. Cells were primed with ultra-pure LPS (100 ng/ml, Invivogen) for 5-6 h after which medium was removed and different concentrations of activators in DMEM were added and left overnight (or 1h for ATP). Potential inhibitors were added 1h before the addition of stimulators. The concentration of secreted IL-1β was measured by ELISA (e-Bioscience) according to manufacturer's instructions.

Western blotting. Experiment were performed as for measuring IL-1β ELISA, with exception that stimulation was finished after 3 h. Methanol precipitation was used to precipitate proteins from cell culture media. Cells were washed twice with cold PBS and lysed. Protein concentration in cell lysate was measured with BCA. Proteins were separated on 15% SDS-PAGE gels, blotted onto the nitrocellulose membrane (GE Healthcare) and detected with appropriate primary and secondary antibodies (for detection of IL-1β: B122 and Goat anti-

Armenian hamster IgG-HRP (Santa Cruz), for detection of caspase-1: M-20 (Santa Cruz) and goat anti-mouse HRP-conjugated secondary antibodies from Jackson Immunoresearch). SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) was used for detection of HRP-labeled bands.

Caspase-1 activation. Activation of caspase-1 was followed under the confocal microscope Leica and flow cytometer Cyflow (Partec) using Fluorochrome Inhibitor of Caspase 1 kit (Immunochemistry Technologies) according to manufacturer's instructions.

Confocal microscopy. A Leica TCS SP5 laser scanning microscope mounted on a Leica DMI 6000 CS inverted microscope (Leica Microsystems, Germany) with an HCX plan apo 63× (NA 1.4) oil immersion objective was used for imaging. For acquisition and images processing, Leica LAS AF software was used.

To follow phagocytosis of fibrils conjugated with Alexa 633, fibrils were first labeled with Alexa Fluor 633 (Invitrogen) according to manufacturer's instructions. Macrophages were primed with LPS (100 ng/ml) and fibrils-Alexa 633 conjugate was added several hours prior staining with Cholera toxin subunit B Alexa 488 conjugate (1 mg/l) (Invitrogen) and Hoechst (1 mg/l). Cells were washed and fixed with 4% PFA. To follow effect of fibrils upon lysosomes, Oregon green dextran (10 mg/l) or DQ-ovabumin (10 mg/l) (both from Invitrogen) were added to primed macrophages together with unlabeled fibrils several hours prior staining with Cholera toxin subunit B Alexa 555 conjugate and Hoechst.

To assess microglia-induced neurotoxicity of PrP fibrils the experiment was performed as described previously (Halle et al., 2008). Mouse neuronal cells CAD (Qi et al., 1997) were first differentiated by withdrawal of serum for 2 days. Microglial cells were added, primed with LPS and stimulated with fibrils. The co-culture was grown in serum-free media for three days. After that cells were fixed with 4% PFA and permeabilized with 0.01% Triton X-100 and stained for neuron-specific marker III β-tubulin (primary antibody Cell Signaling Technologies; secondary antibodies Cy2-labeled goat anti-mouse polyclonal antibodies (Abcam) and with

anti-mouse CD11b eFlour 650 NC antibodies (eBioscience) and observed under confocal microscope.

A 405 nm laser line of 20 mW diode laser was used for Hoechst excitation and emitted light was detected between 430 and 490 nm. A 488 nm and 514 nm laser lines of 100 mW argon laser with 3% laser power were used for Oregon green dextran, Cholera toxin subunit B Alexa 488 conjugate, DQ-ovabumin and Cy2-labeled goat anti-mouse polyclonal antibodies and emitted light was detected between 500 and 560 nm or 525 and 580 nm. A 543 nm laser line of 1.5m mW HeNe laser was used for excitation of Cholera toxin subunit B Alexa 555 conjugate and emitted light was detected in a window between 560 and 630 nm. A 633 nm laser line of 10m mW HeNe laser was used for excitation of fibrils-Alexa 633 conjugate and anti-mouse CD11b eFlour 650 NC antibodies and emitted light was detected in a window between 640 and 700 nm.

NO assay. After priming with interferon-γ for 1 h, different concentrations of PrP105-125 fibrils were added and left overnight. Nitric oxide production will be measured by Griess reaction.

NF-κB activity. Raw-Blue cells (Invivogen) were seeded into wells containing different concentrations of PrP105-125 fibrils in DMEM not supplemented with FBS. The next day, alkaline phosphatase in cell culture medium was analyzed colorimetrically with Quanti-Blue assay (Invivogen).

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FIGURE LEGENDS

Figure 1: The neurotoxicity of PrP fibrils is increased when neurons are co-cultured with microglia. A) Neurons were cultured either alone (top row) or in the presence of microglia (bottom row) and stimulated by LPS (100 ng/ml, left) or LPS and fibrils (8 μM) (right). Neurons were labeled by antibodies against neuron specific β-tubulin and Cy2-labeled secondary antibodies. Microglia were detected by eFlour 650 NC-labeled antibodies against CD11b. Scale bar represents 50 μm. B) Microglia secrete NO upon stimulation with fibrils from prion protein peptide 105-125 comparable to known stimuli LPS (100 ng/ml) and zymosan (10 μg/ml). Representative of 3 independent experiments is shown. Error bars represent the SD of triplicate wells.

Figure 2: Macrophages and microglia release IL-1β upon stimulation with PrP fibrils. Immortalized macrophages (A) and microglia (B) were primed for 6 h with LPS, after which fibrils (10 μM) were added and IL-1β release was followed with time with IL-1β ELISA. Cell culture medium of untreated, primed only and unprimed cells stimulated with fibrils was assayed 19 h post stimulation with fibrils. Error bars represent the SD of triplicate wells. C) Mature IL-1β (p17) is secreted into the cell culture medium (SN) by macrophages after stimulation by PrP fibrils and ATP (5 mM). Pro-IL-1β is followed in cell lysate (CL). Representative Western blot of 4 experiments is shown. D) PrP105-125 fibrils are able to activate NF-κB pathway similarly to LPS (10 ng/ml) and zymosan (1 μg/ml). Representative of 4 experiments is shown. Error bars represent the SD of triplicate wells.

Figure 3: PrP fibrils activate caspase-1. Fluorescent inhibitor of caspase-1 was used to detect caspase-1 activation by PrP fibrils by confocal microscopy (A) or by flow cytometry (B). A) LPS-primed immortalized macrophages were treated with fibrils (10 and 25 μM). Scale bar represents 25 μm. B) Histogram of fibril-treated (10 μM) (full line), primed-only (dash/dotted line) and untreated macrophages (dotted line). The histogram of unlabelled cells (without fluorescent inhibitor of caspase-1) is shown in gray. C) Cleaved caspase-1

(p10) was detected in cell culture media of macrophages stimulated by PrP fibrils and ATP (5 mM). No p10 is detected in untreated or primed only samples. D) IL-1β processing induced by PrP fibrils (20, 10, 7, 5, 3 μM) in immortalized microglia, deficient in caspase-1 and IL-1R and corresponding wild-type microglia was followed by IL-1β ELISA. Secreted IL-1β concentration was corrected for background (priming only). E) IL-1β maturation by PrP fibrils (8.5 μM) in macrophages is decreased upon addition of caspase-1 inhibitor Z-YVAD-FMK. Representative of 3 (A), 2 (B), 3 (C), 3 (D), 3 (E) independent experiments is shown. Error bars represent the SD of duplicate (D) and triplicate (E) wells.

Figure 4: Prion fibrils activate NLRP3 inflammasome. A) Wild-type, NLRP3-deficient and ASC-deficient macrophages were primed and stimulated with different concentrations of PrP fibrils (10, 7, 5, 3 μM). Secreted IL-1β concentration was corrected for background (priming only). B) Apyrase (0.4 U), an ATP hydrolyzing enzyme, had no affect on IL-1β release induced by PrP fibrils (8.5 μM). C) The inhibition of K^+ efflux by addition of 130 mM KCl to medium or by glyburide inhibits IL-1β release by PrP fibrils (20 μM). Representative of 3 (A), 4(B), 4 (C) independent experiments is shown. Error bars represent the SD of triplicate wells.

Figure 5: Fibrils cause lysosome destabilization upon phagocytosis. A) Alexa 633-labeled fibrils (red) are phagocytosed by macrophages (green: cholera toxin subunit B; blue: Hoechst). Scale bar represents 25 μm. B) IL-1β release by PrP fibrils (8.5 μM) is decreased upon addition of phagocytosis inhibitor cytochalasin D. Error bars represent the mean SD of quadruplicate wells. C) Macrophages were incubated with unlabeled fibrils (right) or without fibrils (left) and with DQ-ovalbumin. Lysosome swelling was observed in cells treated with PrP fibrils (right). Scale bars represent 10 μm. D) Cytosolic DQ-ovalbumin stain indicating leakage of lysosomal content into the cytosol was observed in several cells treated with PrP fibrils (red: cholera toxin subunit B, green: DQ-ovalbumin, blue: hoechst). Scale bar represents 10 μm. Representative of 2 (A, C, D) and 4 (B) experiments is shown.

Figure 6: Fibrils are the most potent inflammasome activators among different PrP species. A) Aggregates, B) fibrils, C) oligomers, D) PrP monomers (α-PrP) were visualized under the confocal microscope (aggregates) or AFM and tested for their ability to induce IL-1β release by macrophages. Representative of 3 independent experiments is shown. Error bars represent the SD of triplicate wells. Scale bars represent 20 μm (A), 1 μm (B), 200 nm (C) and 100 nm (D).

SUPPLEMENTARY FIGURE LEGENDS

Supplementary figure 1: PrP fibril-activated microglia induce neuronal death. Neurons and microglia were cultured alone or in a co-culture. LPS was added to all wells. After three days of incubation with or without fibrils (8 μM) toxicity was estimated by an XTT assay, which measures activity of mitochondria enzymes. Absorbance at 490 nm is represented on the ordinate axis. Representative of 2 independent experiments is shown. Error bars represent the SD of triplicate wells.

Supplementary figure 2: PrP fibrils are toxic to macrophages at higher concentrations. After overnight treatment of primed macrophages with different concentrations of PrP fibrils toxicity was assessed by XTT assay. Values were normalized to the fibril-untreated control. Error bars represent the SD of triplicate wells.

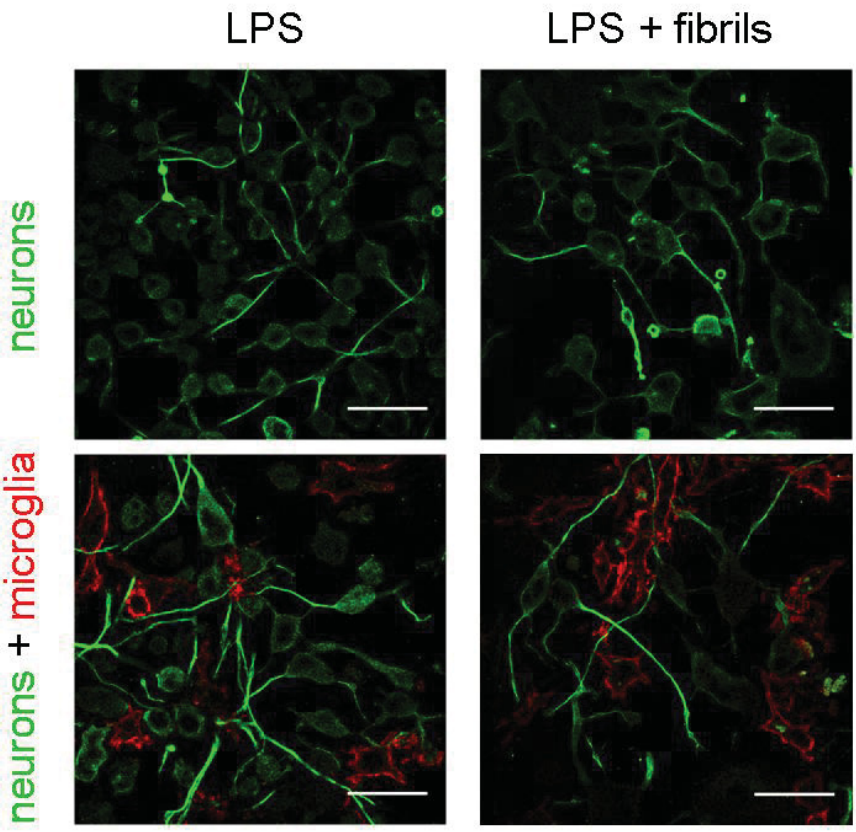
Supplementary figure 3: Appearance of enlarged lysosomes upon phagocytosis of PrP fibrils. Primed macrophages were incubated without fibrils (A) or with unlabeled fibrils (B) and with Oregon green dextran (green). Membrane structures were labeled by cholera toxin subunit B (red) and nuclei wit Hoechst (blue). Scale bar represents 25 μm. Representative of 2 independent experiments is shown.

Supplementary figure 4: Characterization of different PrP forms. A) Far-UV CD spectrum of the monomeric α-form exhibits two minima at 208 nm and 220 nm, which is characteristic of an α-helical secondary structure, as expected for normal PrP. B) Far-UV CD spectrum of PrP fibrils has one minimum at 218 nm, which is characteristic of a β-secondary

structure. C) The formation of oligomeric PrP was analyzed by size-exclusion chromatography. The relative yield of oligomeric PrP was determined from the area of the peak eluting at 18 min after loading.

Figure 1

A


LPS
LPS + fibrils
neurons
neurons + microglia

B

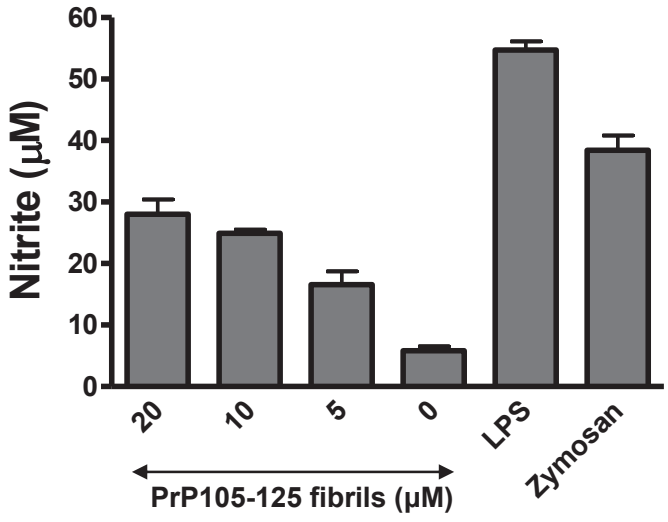

Nitrite (μM)
60
50
40
30
20
10
0
20
10
5
0
LPS
Zymosan
PrP105-125 fibrils (μM)

Figure 2

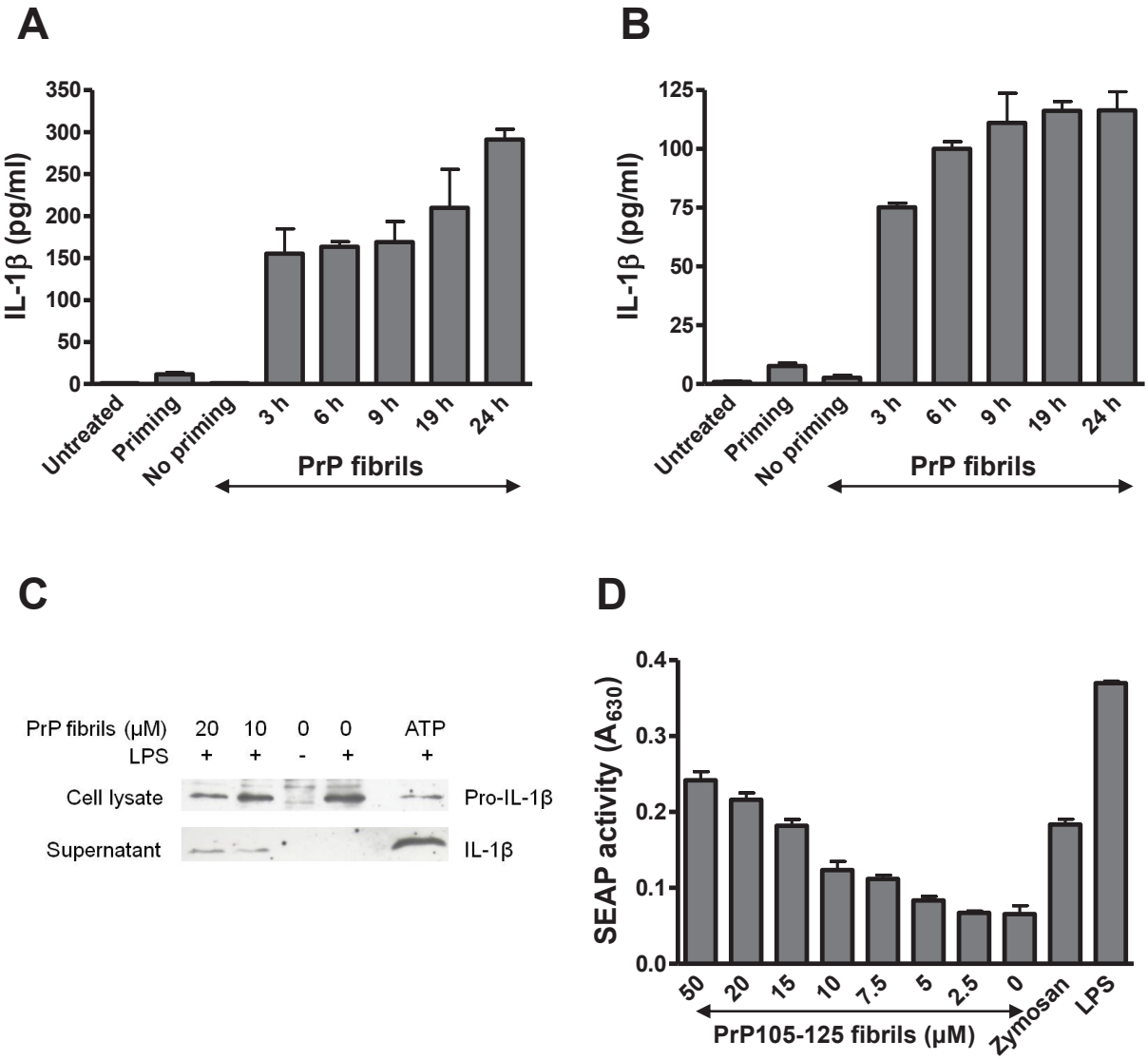

A
IL-1β (pg/ml)
350
300
250
200
150
100
50
0
Untreated
Priming
No priming
3 h
6 h
9 h
19 h
24 h
PrP fibrils
B
IL-1β (pg/ml)
125
100
75
50
25
0
Untreated
Priming
No priming
3 h
6 h
9 h
19 h
24 h
PrP fibrils
C
PrP fibrils (μM) 20 10 0 0 ATP
LPS + + - + +
Cell lysate
Pro-IL-1β
Supernatant
IL-1β
D
SEAP activity (A630)
0.4
0.3
0.2
0.1
0.0
50 20 15 10 7.5 5 2.5 0
PrP105-125 fibrils (μM)
Zymosan
LPS

Figure 3

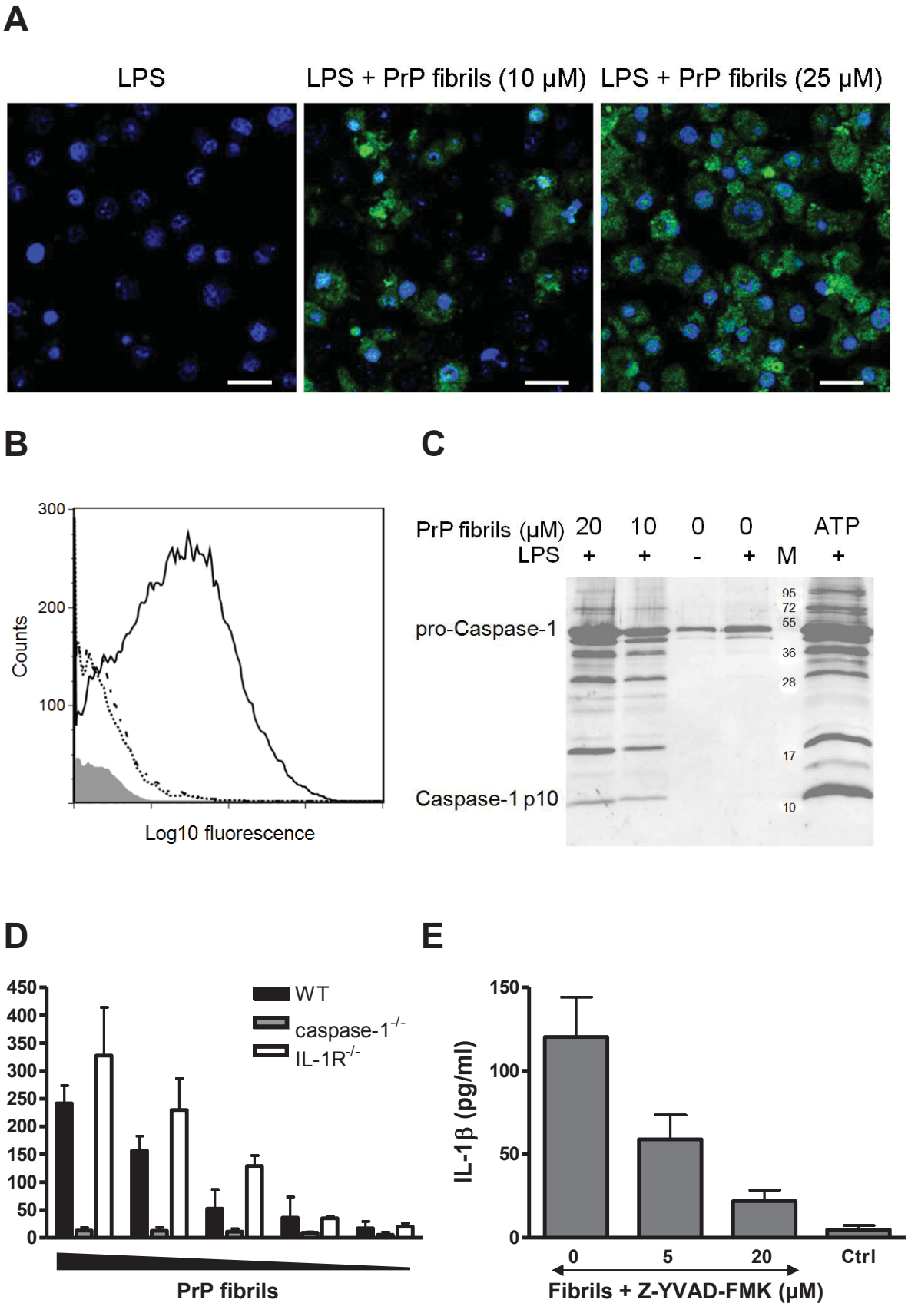

A
LPS
LPS + PrP fibrils (10 µM)
LPS + PrP fibrils (25 µM)
B
300
200
100
Counts
Log10 fluorescence
C
PrP fibrils (µM) 20 10 0 0 ATP
LPS + + - + M +
pro-Caspase-1
Caspase-1 p10
95
72
55
36
28
17
10
D
450
400
350
300
250
200
150
100
50
0
IL-1β (pg/ml)
WT
caspase-1-/-
IL-1R-/-
PrP fibrils
E
150
100
50
0
IL-1β (pg/ml)
0
5
20
Ctrl
Fibrils + Z-YVAD-FMK (µM)

Figure 4

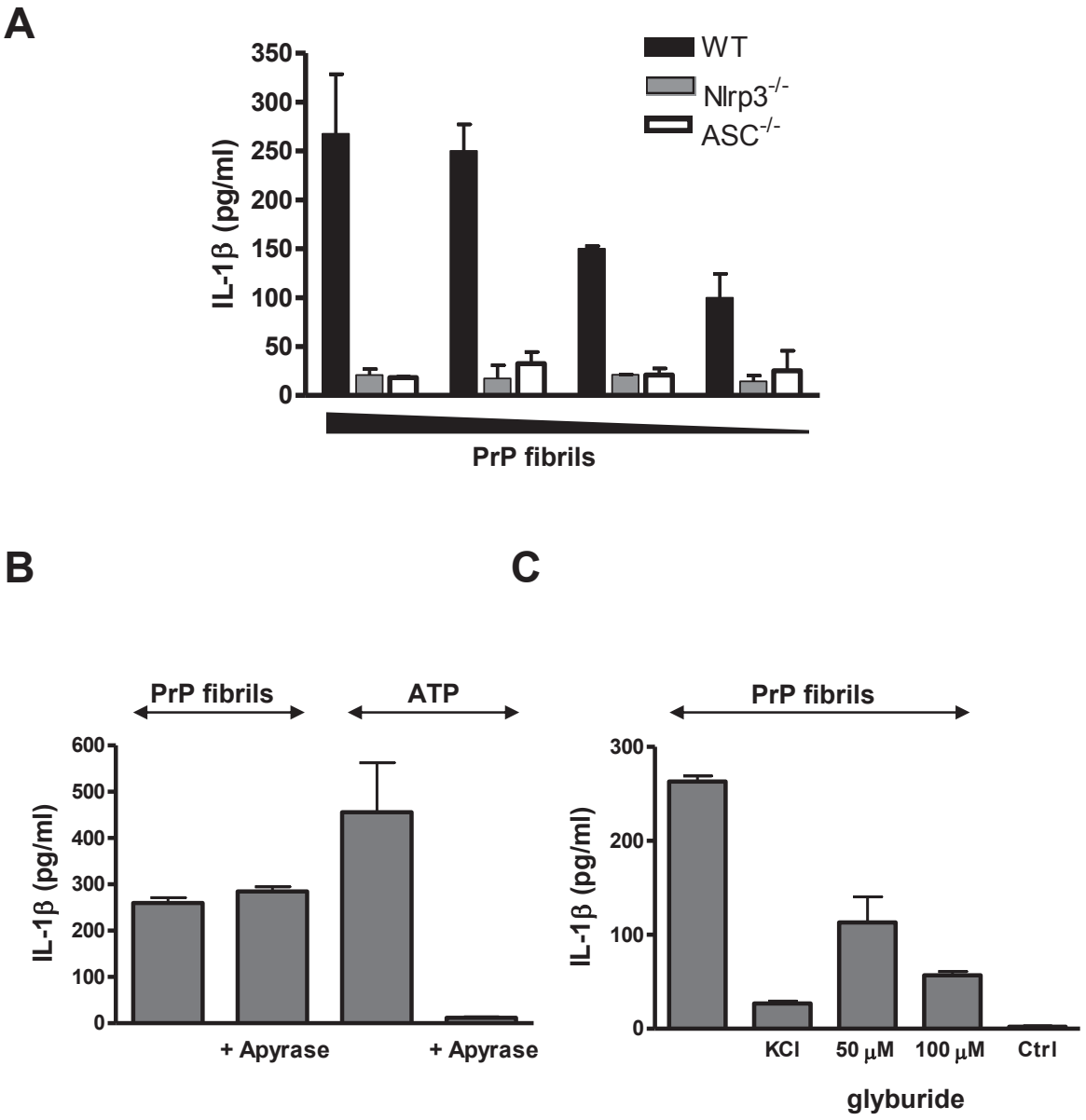

A
WT
Nlrp3-/-
ASC-/-
IL-1β (pg/ml)
350
300
250
200
150
100
50
0
PrP fibrils
B
PrP fibrils
ATP
IL-1β (pg/ml)
600
500
400
300
200
100
0
+ Apyrase
+ Apyrase
C
PrP fibrils
IL-1β (pg/ml)
300
200
100
0
KCl
50 μM
100 μM
Ctrl
glyburide

Figure 5

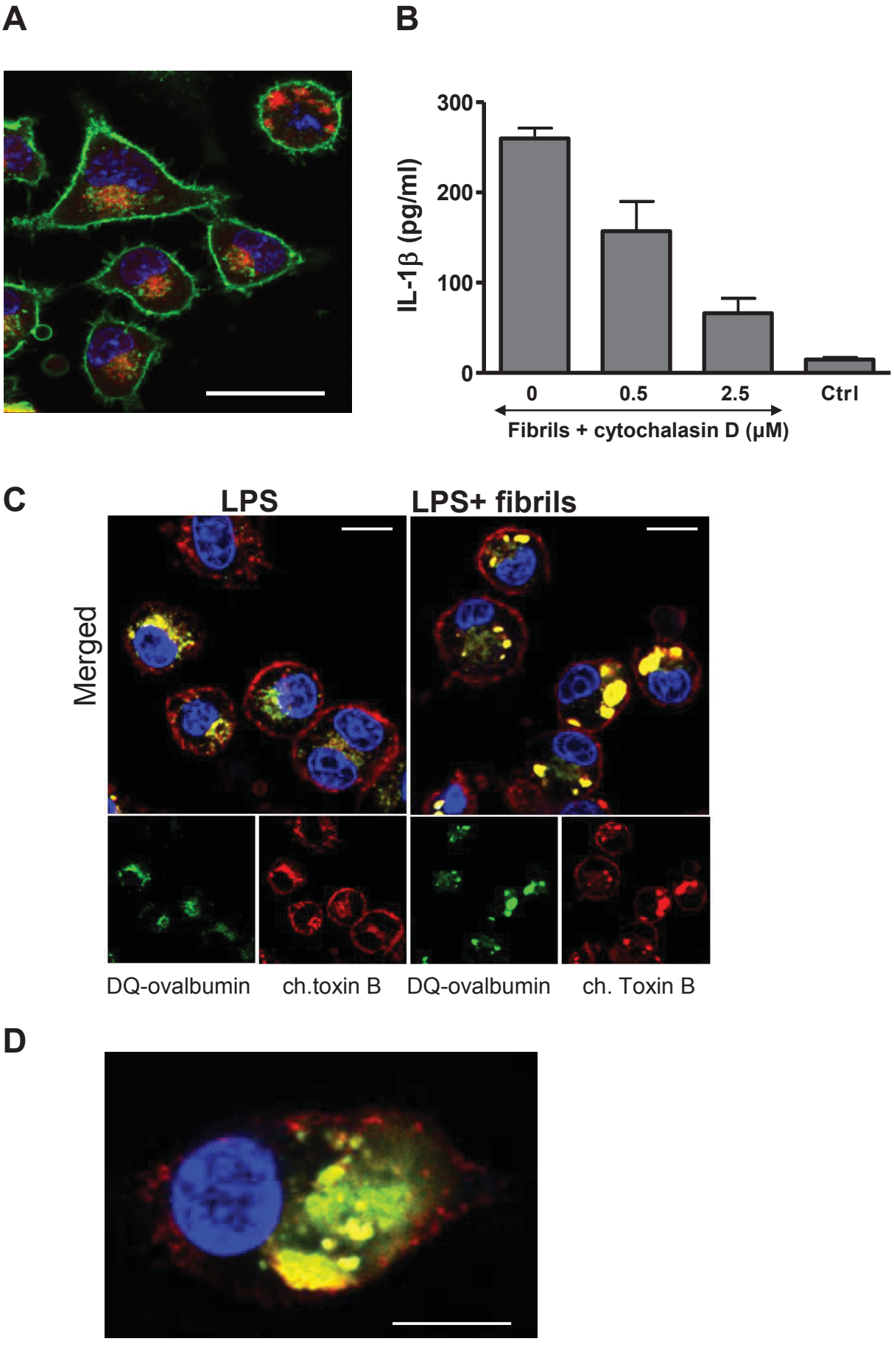
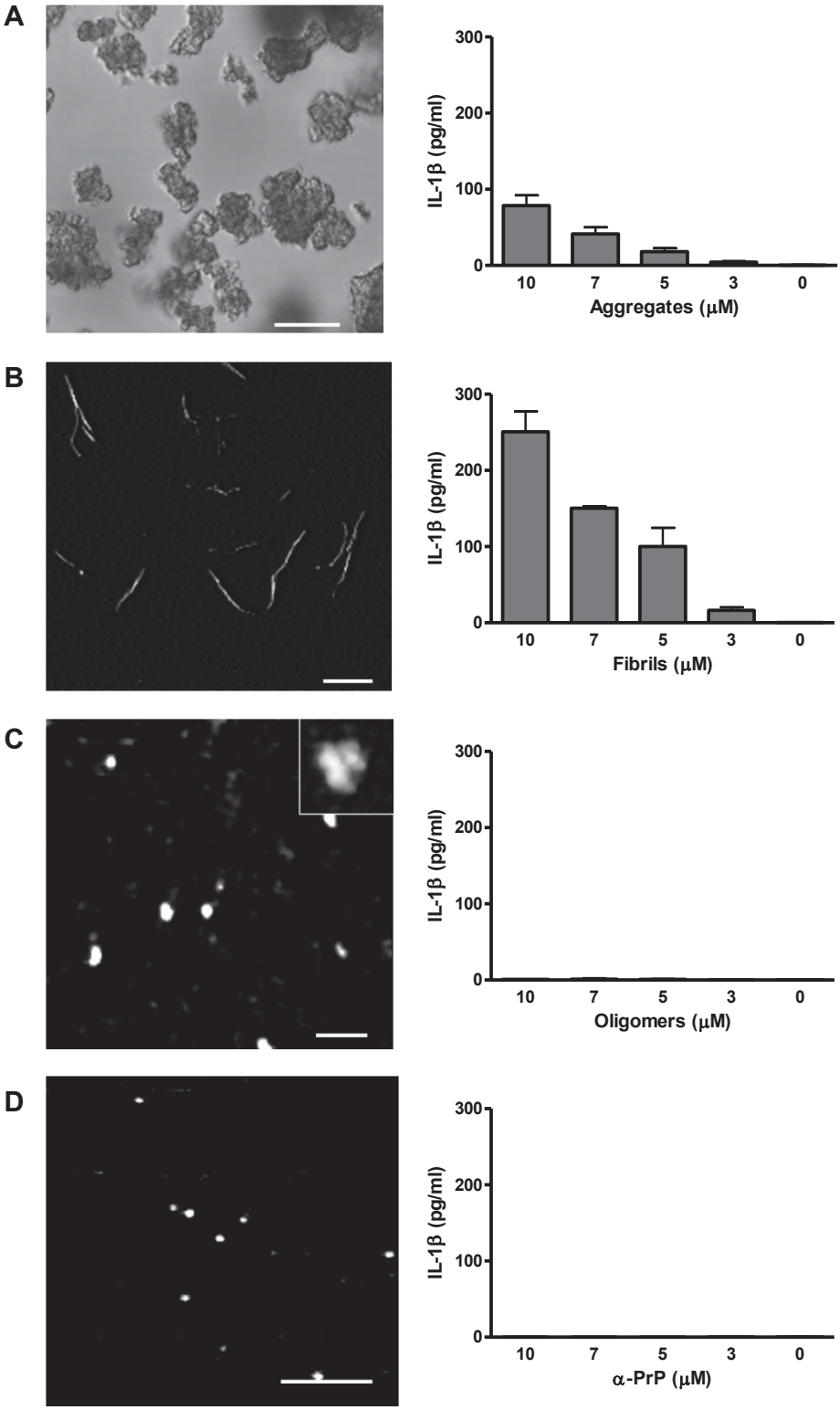
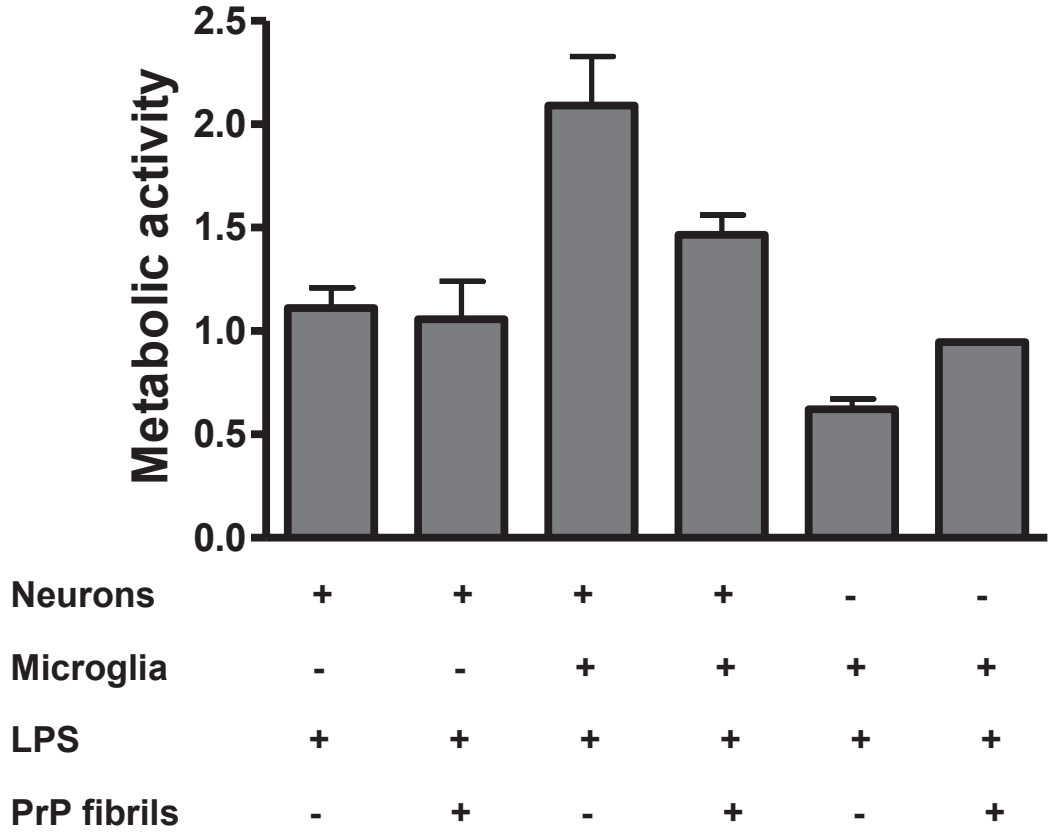

A
B
300
200
100
0
IL-1β (pg/ml)
0
0.5
2.5
Ctrl
Fibrils + cytochalasin D (μM)
C
LPS
LPS+ fibrils
Merged
DQ-ovalbumin
ch.toxin B
DQ-ovalbumin
ch. Toxin B
D

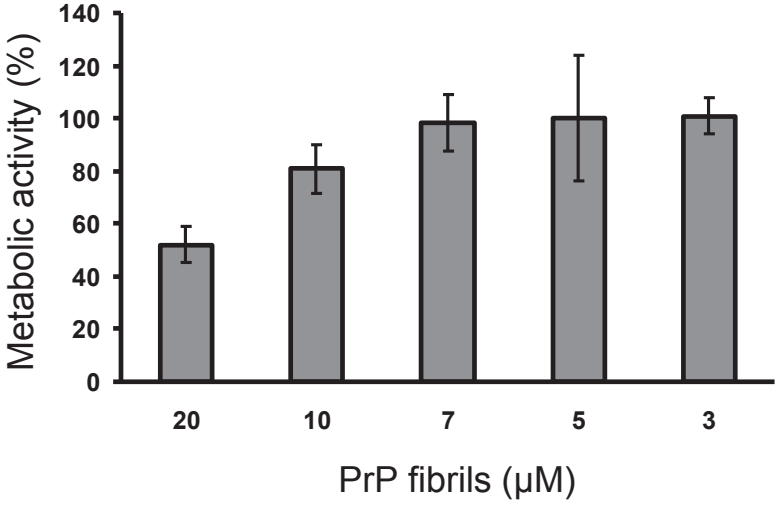
Figure 6


A
B
C
D
300
200
100
0
IL-1β (pg/ml)
10
7
5
3
0
Aggregates (μM)
Fibrils (μM)
Oligomers (μM)
α-PrP (μM)

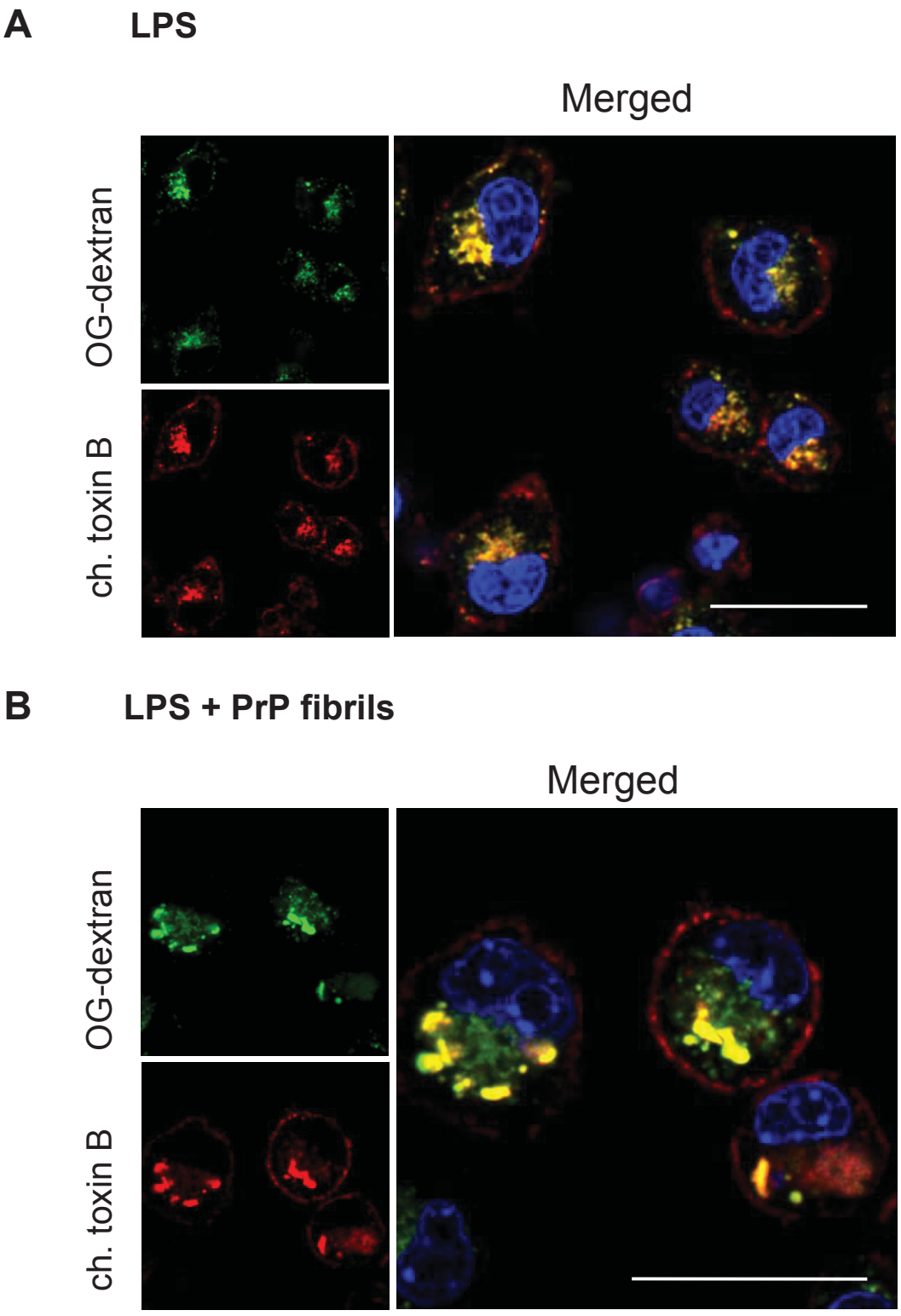
Supplementary figure 1



Supplementary figure 2



Supplementary figure 3



Supplementary figure 4