# English Summary
# Dutch Summary
# Portfolio
# Curriculum Vitae
# Acknowledgements

## ENGLISH SUMMARY

Our immune system consists of a complex network of multiple players. These serve to keep foreign pathogens away, while tolerating our own proteins or harmless organisms. In other words, the immune system requires a balance between immunity on one hand, and tolerance on the other hand. Excessive activation can tip the balance into the domain of autoimmune disorders. Typical autoimmune disorders are rheumatoid arthritis, inflammatory bowel disease (IBD), psoriasis and systemic lupus erythematosus (SLE). It is estimated that within Europe and USA 6-7% of the population has a diagnosed autoimmune disorder. Studying the pathogenesis of autoimmune disorders has been an ongoing quest, as many immune cells take part in it.

Genome-wide association studies (GWAS) reveal a wide range of genes that could be implicated in autoimmune disorders. One of these genes, TNFAIP3 (A20), is associated to multiple autoimmune disorders and is a protein that inhibits the NF-κB pathway that is essential for the activation and survival of many immune cells. In simple terms, A20/TNFAIP3 is one of the most important brake-mechanism protein on immune cell activation.

A unique immune cell, discovered in the 1970s by Nobel laureate Ralph Steinman, termed dendritic cells, is the prime orchestrator of the balance of the immune system. By genetic engineering in mice, the *Tnfaip3* gene could specifically be removed from DCs, which resulted in their activation. This led to activation of several other primary immune cells such as T cells and B cells, and a phenotype that resembled human autoimmune diseases. Our group saw a phenotype resembling SLE, while another research group documented a phenotype of inflammatory bowel disease.

In **chapter 2** we highlight all mouse models known to date in which a targeted deletion of the *Tnfaip3* gene was performed in different immune cells that are known to be involved in autoimmune disorders. We summarize small DNA mutations (single nucleotide polymorphisms (SNPs)) in the human A20/TNFAIP3 locus that have functional and therapeutic consequences for autoimmune patients.

While the population of DCs became more defined over the course of the last 10 years, we utilized a specific Cre-LoxP model (Dngr1-cre) to delete the *A20/Tnfaip3* gene from the conventional type 1 DC (cDC1s) (**Chapter 3**). We found that cDC1s were also activated in these mice, and that by the age of ~31 weeks they developed autoimmune liver inflammation. T cells and B cells were activated, which resulted in antibody producing cells (plasma cells) that made auto-antibodies of the IgA isotype to components of liver cells.

Another highlight in autoimmune research was the unraveling of the Th17/IL-23 axis, which is important in autoimmune diseases such as arthritis, IBD and psoriasis, but also in other chronic diseases such as asthma. In a house dust mite (HDM)-driven model of airway inflammation, our group demonstrated previously that deficiency of Tnfaip3/A20

in myeloid cells lead to neutrophilic rather than eosinophilic airway infiltration, which condition might resemble therapy-resistant asthma patients. We determined whether neutrophilic infiltrates would be reduced in the absence of IL-17 receptor A (IL-17RA) signaling (**Chapter 4**), because IL-17 is known to control neutrophil attractants such as the CXCL1 chemokine production by airway epithelial cells. Surprisingly, this did not seem to be the case, as other cytokines including IL-1β, IL-23 and GM-CSF, which also have the capacity to induce neutrophilic-attracting chemokines, were still produced. We also determined whether the arthritis-like phenotype in mice that lack Tnfaip3 in myeloid cells was reduced in the absence of IL-17RA-signaling (**Chapter 4**). The paw inflammation seen in these aged mice was not dependent on IL-17, but was later demonstrated by another group to be mostly driven by IL-1β.

When studying *Tnfaip3*$^{CD11c\text{-}KO}$ mice, that developed an SLE-like phenotype, we noticed a reduction of B cells in the spleens of aged mice. This could be explained by a development disorder of B cells in the bone marrow. We thus examined all developmental stages of B cells in young and aged mice in **Chapter 5.** We found that B cell development in the bone marrow was hampered at the immature B cell stage in 6-week-old *Tnfaip3*$^{CD11c\text{-}KO}$ mice and at the pre-B cell stage in 24-week-old *Tnfaip3*$^{CD11c\text{-}KO}$ mice. The developmental disorder might well explain the reduced numbers of mature B cells in the periphery of aged *Tnfaip3*$^{CD11c\text{-}KO}$ mice. Using *in vitro* studies, we determined that systemic effects of DC activation leading to changes in the cytokine milieu in bone marrow was most likely not responsible for the observed defect in B cell development. Rather, the observed age-dependent developmental arrest of B-lineage cells most likely reflected changes in non-B cells, most likely A20/Tnfaip3-deficient DCs in the bone marrow. This indicated that activated DCs in a different compartment than spleen, such as bone marrow, could also hamper B cell development and perhaps their function. Interestingly, B cells that reached the periphery were more easily activated compared to B cells from healthy mice.

Antibody producing plasma cells are derived from B cells. The normal route of B cell activation is as follows: DCs activate T cells, and they in turn activate B cells. However, in certain circumstances DCs can also directly activate B cells without the help of T cells. It had been previously demonstrated that A20/Tnfaip3-deficient bone marrow DCs could activate B cells in *in vitro*, independently of T cell help. In **Chapter 6**, we determined whether T cell-independent activation of B cells also occurs in a mice *in vivo,* if we would disable T-B cell communication by abrogating CD40L expression in mice lacking A20 in dendritic cells. CD40L is an essential protein expressed on activated T cells that is essential for their capacity to support B cell activation. Although T-B cell communication was required to achieve germinal centers and IgG1 antibody production, we found that antibodies of the IgA subclass could still be formed. Kidney glomerular basement membrane thickening was also seen, despite the absence of IgG induction, possibly facilitated by IgA depositions that we could demonstrate in the kidneys of a consider-

able fraction of CD40L-deficient mice with CD11c-Cre-driven deletion of the *A20/Tnfaip3* gene.

Since Th17 cells are so important in autoimmunity, and their development is facilitated by IL-23, we wondered how Th17 cell homeostasis would be affected if we induce IL-23-deficiency in mice with abrogated Tnfaip3 expression from DCs (**Chapter 7**). Surprisingly, Th17 cell homeostasis was not altered in absence of IL-23. Levels of immunoglobulins, autoreactive immunoglobulins and kidney glomerular changes were also unaltered in these mice. We thus concluded that the SLE phenotype seen in mice with Tnfaip3-deficient DCs was independent of the Th17/IL-23 axis.

Taken together, DCs maintain the balance between tolerance to autoimmunity. The A20/Tnfaip3 protein in DCs helps in keeping the balance: loss of this protein tips the immune system into a state of autoimmunity. By utilizing several genetic mouse models of targeted A20/Tnfaip3 deletion from specific DC subsets, we have seen that the absence of A20/Tnfaip3 can result in different autoimmune phenotypes, e.g. an autoimmune liver phenotype or SLE. In more detail, the SLE phenotype is quite robust, because despite inhibiting the Th17/IL-23 axis or T-B cell communication, the mice still developed a disease phenotype with characteristics of SLE. This highlights that future of therapies may need to be targeted to the start of an autoimmune reaction, for example at the level of DCs, because at a later point in disease development many other immune players and cytokines are involved, perhaps irreversibly. Those future therapies will be crucial to help DCs to maintain their *Act of balance*.

## NEDERLANDSE SAMENVATTING

Ons immuunsysteem bestaat uit een complex netwerk van verschillende immunologische spelers. Zij werken samen om vreemde lichamen zoals virussen en bacteriën te weren, terwijl ze andere eiwitten of organismen welke ongevaarlijk zijn moeten tolereren. Met andere woorden: ons immuunsysteem heeft een balans nodig tussen immuniteit aan de ene kant en tolerantie aan de andere kant. Te veel activatie van het immuunsysteem leidt tot gezondheidsproblemen op het gebied van auto-immuunziekten, waarin lichaamseigen eiwitten worden aangevallen. Karakteristieke auto-immuunziekten zijn reumatoïde artritis, inflammatoire darm aandoeningen (IBD), psoriasis en systemische lupus erythomatodes (SLE). Naar schatting heeft 6 à 7% van de Europese en Amerikaanse bevolking een auto-immuun aandoening. Onderzoek naar het ontstaan van auto-immuunziekten is een zoektocht die al tientallen jaren speelt vanwege de complexiteit van de vele immuuncellen die er aan deelnemen.

Door genetisch onderzoek via genoombrede associatie studies zijn vele genen gevonden die betrokken kunnen zijn bij auto-immuun aandoeningen. Één van die genen is TNFAIP3 (ook bekend als A20), welke geassocieerd is met verscheidene auto-immuun ziekten. Het is een eiwit dat de NF-κB signaleringsroute remt, die zeer belangrijk is voor cel activatie en overleving. Eenvoudig gezegd: TNFAIP3/A20 is één van de belangrijkste regulatoren die als rem functioneert in immuuncel activatie.

Eén unieke immuuncel, de dendritische cel (DC), ontdekt in de jaren '70 door Nobelprijswinnaar Ralph Steinman, is de dirigent van het immuunsysteem. Door middel van extra signalen op het celoppervlak (co-stimulatoire en co-inhibitoire moleculen) kan de DC bepalen of een volgende immuuncel, de T cel, geactiveerd wordt. Met behulp van genetische muismodellen is het mogelijk om Tnfaip3/A20 specifiek weg te halen in een enkel celtype, bijvoorbeeld de DC. Dit leidt dan tot spontane activatie van deze DCs. Als je deze muizen, die A20/Tnfaip3 missen uit DCs (deze zijn *Tnfaip3*$^{CD11c\text{-}KO}$ muizen genoemd), oud laat worden treedt activatie op van andere immuuncellen, zoals T en B cellen. Als gevolg daarvan ontwikkelen deze dieren een ziektebeeld dat grote overeenkomsten vertoont met autoimmuunziekten bij de mens. Onze groep heeft aangetoond dat *Tnfaip3*$^{CD11c\text{-}KO}$ muizen SLE-achtige verschijnselen ontwikkelen, terwijl een andere onderzoeksgroep heeft laten zien dat vergelijkbare muizen een IBD ziektebeeld kunnen ontwikkelen. In **Hoofdstuk 2** geven we een overzicht van de nu bekende muismodellen waarin A20/Tnfaip3 specifiek is verwijderd uit verschillende immunologische cellen die een rol spelen bij autoimmuun reacties. We noemen ook de kleine DNA variaties (de zgn. enkel-nucleotide polymorfismen of SNPs) die bij patiënten worden gevonden en een mogelijke verklaring kunnen zijn voor functionele veranderingen in hun immuunsysteem en mogelijk therapeutische consequenties hebben.

Doordat de populatie van DCs steeds beter gedefinieerd werd in de laatste 10 jaar, werd ook steeds duidelijker dat er verschillende DC typen waren. Onze interesse viel op een nieuw genetisch model om A20/Tnfaip3 specifiek te verwijderen uit een subtype van DCs, de zgn. conventioneel type 1 dendritische cel (cDC1) (**Hoofdstuk 3**). We zagen in deze *Tnfaip3*$^{Dngr1\text{-}KO}$ muizen dat de cDC1s inderdaad geactiveerd raakten en dat rond de leeftijd van 31 weken deze muizen een autoimmuun leverontsteking hadden ontwikkeld. T cellen en B cellen waren geactiveerd en er waren antistof producerende cellen (plasma cellen) aantoonbaar, die antistoffen van het IgA isotype tegen lichaamseigen levereiwitten produceerden.

Nog een belangrijke ontwikkeling in het onderzoek over auto-immuun ziekten was de ontdekking van de Th17 cel, die de belangrijke signaalstof, het IL-17 cytokine, produceert. De zgn. Th17/IL-23 as van inflammatoire cytokinen en de Th17 cel bleek betrokken te zijn bij auto-immuun ziekten zoals reuma, IBD en psoriasis, maar ook bij andere chronische aandoeningen zoals astma. In een huisstofmijt-geïnduceerd model van luchtwegontsteking in muizen met myeloïde cel-specifieke deficiëntie van A20/Tnfaip3 (*Tnfaip3*$^{LysM\text{-}KO}$) heeft onze groep eerder aangetoond dat muizen een neutrofiel infiltraat in de longen ontwikkelden in tegenstelling tot een eosinofiel infiltraat. Een dergelijk ziekteprofiel met een neutrofiel infiltraat in de longen vertoont overeenkomsten met het immunologisch profiel van patiënten met moeilijk te behandelen astma. We vroegen ons af of de aantrekking en opeenhoping van neutrofielen zou afnemen als we de signaalstof IL-17 zouden wegnemen, aangezien IL-17 betrokken is bij de productie van stoffen die neutrofielen aantrekken zoals het CXCL1 chemokine dat door de cellen van de luchtwegwand wordt geproduceerd onder invloed van IL-17 (**Hoofdstuk 4**). In tegenstelling tot onze verwachting bleek dit niet het geval te zijn, aangezien andere cytokinen zoals IL-1β, IL-23 en GM-CSF nog steeds geproduceerd werden en deze ook in luchtwandcellen de productie van chemokinen die neutrofielen aantrekken kunnen stimuleren. We hebben in deze myeloid-specifieke Tnfaip3-deficiënte *Tnfaip3*$^{LysM\text{-}KO}$ muizen ook gekeken naar een reuma-achtig ziektebeeld dat door een andere onderzoeksgroep was beschreven, en onderzocht of dergelijke ziekteverschijnselen zouden afnemen in afwezigheid van IL-17 receptor signalering. Dit bleek ook niet het geval te zijn. Later werd aangetoond in een ander onderzoek dat cytokine IL-1β hoofdverantwoordelijk is voor het reuma-achtig beeld in myeloid-specifieke Tnfaip3-deficiënte muizen.

Tijdens het bestuderen van de muis welke dendritische cel-specifiek Tnfaip3-deficient is (*Tnfaip3*$^{CD11c\text{-}KO}$ muizen) en een SLE-achtig beeld ontwikkeld, viel het ons op dat de B cellen aanzienlijk verminderd waren in de milt. Omdat deze bevinding wijst op een defect in de aanmaak van B cellen in het beenmerg hebben we in **hoofdstuk 5** de ontwikkeling van B cellen onderzocht in zowel jonge als oudere *Tnfaip3*$^{CD11c\text{-}KO}$ muizen. Er bleek een leeftijdsafhankelijke B cel ontwikkelingsstoornis te zijn, gekenmerkt door een sterke blokkade op het immature B cel en het eerdere pre-B cel stadium in, respec-

tievelijk, 6 en 24 weken oude *Tnfaip3*$^{CD11c\text{-}KO}$ muizen. Deze stoornis is een aannemelijke verklaring voor de afname van B cellen in perifere lymphoïde organen zoals de milt op oudere leeftijd. Door laboratoriumproeven in een beenmerg celkweek systeem hebben we kunnen aantonen dat circulerende signaalstoffen waarschijnlijk niet de afwijking in B cel ontwikkeling probleem induceren, maar dat Tnfaip3-deficiëntie DCs in het beenmerg zelf waarschijnlijk deze afwijkingen in de B cel ontwikkeling veroorzaken. Een andere interessante bevinding was dat volgroeide B cellen die het beenmerg verlaten, wel gemakkelijker geactiveerd raken op eenzelfde stimulus dan B cellen uit een normale gezonde muis. Onze proeven laten zien dat de geactiveerde DCs in ons muis model ook in een ander orgaan dan de milt, zoals dus in het beenmerg, B cel ontwikkeling kan verstoren en mogelijk hun uiteindelijke functie kan beïnvloeden.

Normaalgesproken verloopt de activatie van B cellen via antigeen presenterende cellen zoals DCs, die T cellen activeren, die vervolgens hulp bieden aan B cellen in nauwe B-T cel interactie. Er is echter aangetoond dat Tnfaip3-deficiënte DCs, zonder de hulp van T cellen, in een celkweeksysteem (*in vitro*) B cellen konden activeren. In **hoofdstuk 6**, vroegen we ons af of deze directe activatie van B cellen door Tnfaip3-deficiënte DCs ook in de muis (*in vivo*) plaatsvindt. Om dit te onderzoeken hebben we T en B cel communicatie geblokkeerd door genetisch tevens CD40L weg te halen (een eiwit dat door de T cel na activatie op het celoppervlak wordt gezet en dat belangrijk is voor functionele T-B cel interactie). Deze T-B cel communicatie bleek essentieel te zijn voor het ontwikkelen van kiemcentrum B cellen, een subgroep van B cellen die zich bevinden in kiemcentra waar B cellen na activatie een interactie aangaan met T cellen om vervolgens verder te kunnen uitrijpen o.a. tot antistof-producerende plasma cellen. In de afwezigheid van CD40L werd er geen IgG1 gevormd, maar vonden we dat een andere antistof, IgA, wel gevormd kon worden. Het circulerende IgA in het serum bevatte ook reactiviteit tegen lichaamseigen stoffen. Structurele veranderingen in de glomeruli – dit zijn de kluwens van haarvaatjes die belangrijk zijn voor de bloedfiltratie in de nier – die passen bij een SLE beeld, werden nog steeds aangetroffen ondanks de afwezigheid van IgG.

Omdat Th17 cellen zo belangrijk zijn in auto-immuunziekten en hun ontwikkeling ondersteund wordt door IL-23, vroegen we ons af of de auto-immuun afwijkingen in *Tnfaip3*$^{CD11c\text{-}KO}$ muizen zouden verminderen in de afwezigheid van IL-23 (**Hoofdstuk 7**). In tegenstelling tot de verwachting, was de ontwikkeling van Th17 cellen niet verstoord in afwezigheid van IL-23. Ook het niveau van antistoffen, ook die tegen lichaamseigen eiwitten zoals dubbelstrengs DNA in het serum en afwijkingen in de nieren was bij de IL-23-deficiënte *Tnfaip3*$^{CD11c\text{-}KO}$ niet veranderd ten opzichte van *Tnfaip3*$^{CD11c\text{-}KO}$ die wel IL-23 konden maken. We concludeerden dus dat het SLE fenotype in deze *Tnfaip3*$^{CD11c\text{-}KO}$ muizen onafhankelijk was van de Th17/IL-23 as.

Samenvattend is de DC belangrijk om een goede balans tussen tolerantie en immuniteit te bewaren. Het Tnfaip3/A20 eiwit in DCs speelt hierin een cruciale rol, want als DCs

dit eiwit missen slaat het immuun systeem teveel door richting auto-immuniteit. Door gebruik te maken van verschillende genetische muismodellen waarbij we heel specifiek bepaalde DC subgroepen deficiënt konden maken voor Tnfaip3/A20, zagen we dat deze muizen verschillende auto-immuun ziektebeelden ontwikkelden. Deze varieerden van auto-immuun leverontsteking tot een algehele immuunontsteking die duidelijke overeenkomsten had met SLE. Dit SLE beeld bleek robuust: ook als we T-B cel interactie of de Th17/IL-23 as blokkeerden waren er nog steeds diverse kenmerken van SLE meetbaar. Deze bevindingen benadrukken dat de toekomstige therapie ontwikkeling voor auto-immuunziekten gericht zou moeten zijn op ingrijpen in een vroeg stadium van het ziekteproces, zoals op het niveau van DCs. In een later stadium van de ziekte zijn er veel andere immuuncellen en signaalstoffen betrokken, waardoor het moeilijker wordt een afdoende effect van de behandeling te verkrijgen. Het is dus belangrijk om de DCs te helpen hun activatie strikt te moduleren en hun *act of balance* te ondersteunen.

## PORTFOLIO

### Tridib Das

| | |
|---|---|
| Erasmus MC Department: | Pulmonary medicine |
| PhD Period: | 2013 – 2017 |
| Thesis Directors: | Prof. dr. R.W. Hendriks |
| | Prof. dr. B.N.M. Lambrecht |
| Research School: | Molecular Medicine |

### PhD Courses

2013 Animal Handling Course (MolMed)
2013 Functional imaging and super resolution
2013 Basic Course on R (MolMed)
2014 Advanced Immunology Course (MolMed)
2014 Research Management for PhD Students (MolMed)
2015 Research Integrity Course (MolMed)
2016 Writing a scientific article Course (MolMed)

### (Inter)national conferences

2013 International Symposium on Regulators of Adaptive Immunity (Erlangen, Germany)
2014 ILD Winterschool (Davos, Switserland)
2014 IRC mini-symposium on Cell Signaling in inflammation and immunity (Ghent, Belgium)
2014 NVVI Lunteren (Lunteren, The Netherlands)

### Presentations and posters

2014 MolMed Day (Rotterdam, the Netherlands) – Poster
2014 NVVI 50$^{th}$ Anniversary meeting (Kaatsheuvel, the Netherlands) – Oral
2014 Dendritic Cell conference (Tours, France) – Poster
2015 Keystone Macrophages and Dendritic Cells Re-united (Montreal, Canada) – Poster
2015 MolMed Day (Rotterdam, the Netherlands) – Poster
2016 MolMed Day (Rotterdam, the Netherlands) – Poster
2016 Dendritic Cell conference (Shanghai, China) – Oral

### Coaching, Teaching and management activities

Supervision of Fatemeh Ahmedi (Master student)
Supervision of Anne Hubers (Master student)

Supervision of Zhongli Chen (Master student, guiding in writing a review article)
Mentor for 1st year medical graduate students
Board member of PROMERAS (PhD association Erasmus MC)

**Publications**

**2018** **Das, T.**, Chen, Z., Kool, M. A20/Tumor Necrosis Factor α-Induced Protein 3 in Immune Cells Controls Development of Autoinflammation and Autoimmunity: Lessons from Mouse Models.
*Front Immunol. 2018 Feb 21;9:104*
Impact factor: 4.534

**2018** Vroman, H., **Das, T.**, Bergen, I.M., van Hulst, J.A.C., Ahmadi, F., van Loo, G. Lubberts, E., Hendriks, R.W., Kool, M. House dust mite-driven neutrophilic airway inflammation in mice with TNFAIP3-deficient myeloid cells is IL-17-independent.
*Clin Exp Allergy. 2018 Dec;48(12):1705-1714.*
Impact factor: 4.641

**2019** **Das, T.**, Bergen, I.M., Koudstaal, T., van Hulst, J.A.C., van Loo, G., Boonstra, A., Vanwolleghem, T., Leung, P.S.C., Gershwin, M.E., Hendriks, R.W., Kool, M.
*J Autoimmun. 2019 Aug;102:167-178*
Impact factor: 7.321

## ABOUT THE AUTHOR

Tridib Das was born in New Delhi, India on 14th October 1988. He attended bilingual VWO at the Alfrink College in Zoetermeer, after which he studied medicine from Leiden University. In 2007 he went as an exchange student to the Karolinska University in Stockholm, at which time an interest in biomedical sciences started to grow. This steered him to enroll in a pre-master biomedical sciences at Leiden University. After achieving cum laude on his medicine degree in 2012, he completed his masters in biomedical sciences from Leiden University in 2013, which included a research internship at Tufts and Yale University in Boston and New Haven (USA).

Choosing a clinical career path could change for him every month during his clinical rotations, varying from pediatrician, to ENT-specialist to gynaecologist. His final choice was set for ophthalmology. One passion, however, was continuous throughout his studies and became evident from all his student research internships: the immune system. It intrigued him how it entangled every organ of our human body and was layered in so many different ways. Logically a PhD vacancy on autoimmune disorders at Erasmus MC became his choice for dedicating the next phase of life before starting a clinical residency. With positive responses from Boston through Skype interviews his future supervisors in Rotterdam from the pulmonary medicine department, Tridib jumped aboard the research line in 2013 on the role of A20/Tnfaip3 in dendritic cells to balance autoimmunity. The results of this thesis are now in front of you and were defended in Rotterdam on 24th February 2021.

As per June 2018 Tridib is in his residency to become an ophthalmologist from the Rotterdam Eye hospital. In his spare time he enjoys photography, traveling the world, tasting different cuisines and analyze movies of the silver screen. He lives with his wife Smriti in Rotterdam.

## ACKNOWLEDGEMENTS

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Thankfully, even on the days that nothing in my PhD seemed to go in the right direction, I found solace within the Pulmonology Lab at the Erasmus MC, and made me look forward to go to the lab every morning. The atmosphere at the lab was simply phenomenal. Despite having a large lab, we regularly managed to go for lunch all together. My thanks go out to my fellow PhDs at the time: I had just moved to Rotterdam and felt we had become not only colleagues, but also friends.

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**Paulien**, it was awesome that we independently joined the same Lindy hop dance classes and could checkmark some old-fashioned parties around Rotterdam. Thanks for your ongoing enthusiasm which really brought life to the lab, your co-founded Pulmonology LongDrinks will hopefully be carried on by the youngsters for many years to come.

**Simar**, if it was very late in the evening or sometime in the weekend, I was always sure that besides me there would be one more Indian roaming the lab. Your motivation transferred on to me to keep working hard and making the hours, which now at last pays

off. I am glad you could secure your career in Netherlands and wish you all the best in Nijmegen.

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